



Gangliosides profiling in serum of breast cancer patient: GM3 as a potential diagnostic biomarker

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Abstract

Gangliosides altered during the pathological conditions and particularly in cancers. Here, we aimed to profile the gangliosides in breast cancer serum and propose potential biomarkers. LC-FTMS method was first used to identify all the ganglioside species in serum, then LC-MS/MS-MRM method was employed to quantitate the levels of gangliosides in serum from healthy volunteers and patients with benign breast tumor or breast cancer. 49 ganglioside species were determined, including GM1, GM2, GM3, GD1, GD3 and GT1 species. Compared to healthy volunteers, the levels of GM1, GM2, GM3, GD1 and GD3 displayed a rising trend in breast cancer patients. In particular, as the major glycosphingolipid component, GM3 showed excellent diagnostic accuracy in cancer serum (AUC > 0.9). PCA profile of the GM3 species showed clear distinction between normal and cancer serum. What's more, ROC curve proved great diagnostic accuracy of GM3 between cancer and benign serum. In addition, GM3 was discovered as a diagnostic marker to differentiate luminal B subtype from other subtypes. Furthermore, a positive correlation between GM3 and Ki-67 status of patients was identified. In conclusion, our results introduced the alteration patterns of serum gangliosides in breast cancer and suggested serum GM3 as a potential diagnostic biomarker in breast cancer diagnosis and luminal B subtype distinction.

Keywords Breast cancer · Biomarker · Gangliosides · GM3 · Serum

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. According to the latest statistics from the National Cancer Center, the incidence and mortality of breast cancer rank 1st and 5th among China women malignant tumors, respectively [1, 2]. Risk factors of breast cancer involve

age, family history, marital and parental status [3]. Additional, breast cancer is a molecularly heterogeneous tumor that can be classified into several intrinsic molecular subtypes [4]. According to the expression status of estrogen receptor (ER), progesterone receptor (PR), ERBB2/HER2 and Ki67 detected by immuno-histochemistry, breast cancer can be divided into four subtypes: luminal A (LA) subtype, luminal B (LB) subtype, HER-2 overexpressing (HER) subtype and basal-like (BS) subtype [5–8]. Molecular subtypes of breast cancer have different targeted therapies and different risk of disease recurrence and survival, which can guide appropriately individualized treatment [9–12].

Cell surface glycoconjugates are involved in numerous essential biological activities including cell proliferation, adhesion and migration, cell–cell signal transduction, immune modulation [13]. However, it is now becoming increasingly evident that aberrant glycosylation on the surface of cancer cells has become a common hallmark of pathological conditions including cancer [14, 15]. The altered glycoconjugates during the development of tumors can serve as important biomarkers and provide a set of specific targets for early diagnosis and therapeutic intervention [16, 17]. And nowadays the discovery of

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glycan-based cancer biomarkers or drug targets has become more and more attractive and widely explored for the diagnosis, treatment, and prognosis of tumors. For example, CA19–9 (SLe^a) was considered as prominent biomarker of colon cancer, gastric cancer and pancreatic cancer [18–20]; CA125 (MUC16) was used as biomarker of ovarian cancer [21]; CA15–3 (aberrantly glycosylated MUC1) was recognized to detect breast cancer [22, 23]. However, these current serum biomarkers have common shortcomings of poor specificity, low sensitivity and difficulty to distinguish subtypes.

Gangliosides belong to the sphingolipid family and are distinguished by a ceramide moiety attached to sialic acid containing complex oligosaccharides. Gangliosides play crucial roles in the normal physiological process, such as embryogenesis, cell growth and inflammatory response [24], as well as in pathological conditions and particularly in cancers [25]. Recent studies indicate that gangliosides can promote tumor-associated angiogenesis, regulate cell adhesion/motility and act as modulators of signal transduction, resulting in invasion and metastasis of cancer cells [26, 27]. Fuc-GM1 with a ceramide containing almost exclusively 2-hydroxy fatty acids was demonstrated to be a characteristic ganglioside in small cell carcinoma of the lung [28]. Tsuchida *et al.* suggested that the overexpression of GM2 might be directly related to the tumorigenicity of human melanoma [29]. Additional, GM2 was overexpressed on the cell surface of a number of human cancers, including melanoma, sarcoma, and renal cancer [30]. GD3 or GM2 can promote tumor-associated angiogenesis, thus induce invasive and metastatic of tumor cells [27]. Nohara *et al.* reported an 18-fold increase of the amount of GM3 in MDA MB-231 cells [31]. Silencing of GM3 synthase can suppress cell migration, invasion, anchorage-independent growth, and lung metastasis in murine breast cancer cells [32]. Marquina *et al.* demonstrated the levels of GM3 in breast cancer tissues were 2.8-fold greater than those in normal tissues [33].

Liquid chromatography-Fourier transform mass spectrometry (LC-FTMS) is widely used in the lipidomics study for the high sensitivity, accurate identification and high resolution. Furthermore, the MS-based technique of multiple reaction monitoring (MRM) is now widely used in the biomarker discovery of metabolomics and proteomics for the rapid, sensitive, and specific quantitation of analytes in complex matrices [34]. In this paper, LC-FTMS method was first used to identify all the ganglioside species in serum, then LC-MS/MS-MRM method was employed to evaluate the levels of gangliosides in serum from healthy volunteers and patients with benign breast tumor or breast cancer before treatment. Systematic statistical analysis of different ganglioside levels in serum was investigated to propose potential diagnostic biomarkers in breast cancer.

Materials and methods

Materials and chemicals

Ganglioside standards (including GM1, bovine brain; GM2, human brain; GM3, bovine buttermilk; GD1, bovine brain; GD3, bovine buttermilk; GT1, bovine brain) were obtained from Matreya, LLC (Pleasant Gap, PA, USA). Ammonium acetate and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and isopropanol were of chromatographic grade from Merck (Darmstadt, Germany), while other reagents were analytical grade.

Serum samples preparation and isolation of gangliosides

The patients were recruited from Qingdao Municipal Hospital, China. Sera of thirty female healthy adults, twenty benign breast tumor patients and seventy breast cancer patients were collected as normal, benign and cancer groups respectively. About 2 mL nonhemolytic blood samples, drawn from the cubital vein, were allowed to coagulate at room temperature for 30 min and subsequently centrifuged at 2000×g for 10 min. The supernatant (0.8–1.0 mL serum) was collected, subpackaged and stored at –80 °C for later analysis. The protocol for this study was approved the local Ethics Committee of Qingdao Municipal Hospital and was performed in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants.

Serum samples were naturally thawed at 4 °C before analyzing. The crude lipids in serum were extracted according to the method of Bligh and Dyer [35] with some modification. Briefly, 100 μL of serum was extracted with chloroform/methanol (2:1, 3 mL). The samples were sonicated for 10 min and centrifuged at 8000 rpm for 10 min. 1 mL of water was added to the supernatant. After centrifuging at 8000 rpm for 10 min, the upper layer was filtered. The lower phase was re-extracted twice by methanol/H₂O (2:1, 0.5 mL). The upper phase was gathered and dried by evaporation under nitrogen. Finally, the dried ganglioside extracts were re-dissolved in 80% methanol for LC-MS injection.

Profiling of gangliosides by reversed-phase (RP)-HPLC-FTMS analysis

RP-HPLC-FTMS analysis was performed on an Agilent 1290 LC UPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with a LTQ ORBITRAP XL mass spectrometer (Thermo, SCIENTIFIC, USA). A sample (5 μL) was injected into a reversed-phase packed column (Poroshell 120 EC-C18, 50 mm × 3 mm I.D., 2.7 μm, Agilent, USA). It was eluted at a column temperature of 25 °C and flow rate of 0.2 mL/min, with a binary solvents of 10 mM NH₄Ac in water

(Containing 0.1% acetic acid) (A) and 85% methanol/15% isopropanol (Containing 10 mM NH₄Ac, 0.1% acetic acid) (B). The gradient was programmed as 30% B initially for 3 min, then changed to 100% B rapidly and maintained at 100% B over 10 min. The analysis was performed in the negative ion mode using a capillary temperature of 275 °C. The spray voltage was 4.2 kV and nitrogen dry gas flowed at 40 L/min. Data acquisition and analysis were performed using Xcalibur 2.0 software.

Data acquisition and analysis were performed using Xcalibur 2.0 software (Thermo, Scientific, Waltham, MA, USA). According to the accurate *m/z*, retention time, relative retention time of the species in the same class, and the spectra of tandem mass spectrometry (MS/MS), ganglioside species were confirmed. The relative quantitative information was generated by peak areas of the gangliosides from extracted ion chromatograms.

Quantification analysis of gangliosides by LC-MS/MS-MRM-based methods

LC-MS were performed on a triple quadrupole mass spectrometer (TSQ Quantiva Ultra, Thermo Inc.) running at the MRM mode. The UPLC method was the same as 2.3. The final optimized conditions and collision energies for the gangliosides MRM transitions are listed in Table 1. Mixed ganglioside standards stock solution of GM1, GM2, GM3, GD1, GD3 and GT1 were serially diluted to give a 10-point calibration curve from 0.005 to 10 ng·μL⁻¹. The content of GM1–36:1, GM2–36:1, GM3–41:1, GD1–38:1, GD3–41:1 and GT1–38:1 in ganglioside standards were chosen as quantitative components. The analysis was performed in the negative ion mode using a capillary temperature of 350 °C and polarization temperature of 300 °C. The spray voltage was 3.0 kV. Sheath gas flowed at 30 Arb and auxiliary gas flowed at 2Arb. Data acquisition and analysis were performed using Xcalibur 2.0 software.

Statistical analysis

Statistical analyses of obtained gangliosides data were performed using SPSS v. 22.0 (IBM Corporation, Armonk, NY) and GraphPad Prism v. 5.03 (GraphPad Software, San Diego, CA). Student's *t*-tests were performed with two-tailed distribution to test the alteration between groups. The gangliosides data with statistical significance (*p* < 0.05) were assessed by the receiver operator characteristics (ROC) test using SPSS v. 22.0. Principal component analysis (PCA) was performed using OriginPro 2018 (OriginLab, Northampton, USA) to allow the visualization of multivariate information.

Results

Identification of ganglioside species in serum by RP-HPLC-FTMS

In this paper, RP-HPLC-FTMS was first conducted as a nontargeted method to identify all the ganglioside species in serum. Sera of 20 breast cancer patients and 10 healthy volunteers were analyzed by RP-HPLC-FTMS method under negative ion modes. According to the accurate *m/z*, retention time, relative retention time of the species in the same class, and the spectra of tandem mass spectrometry (MS/MS), ganglioside species were confirmed. Ultimately, 49 ganglioside species were determined and the list of these ganglioside species was provided in Table S1. Among them, GM3 possessed 23 species and occupied the most abundant component. The extracted peak area of gangliosides in Fig. 1 showed significant differences between breast cancer patients and healthy controls (*p* < 0.05). To realize the quantitative analysis, a targeted LC-MS/MS-MRM method was further conducted.

The targeted ganglioside-lipidomics method based on LC-MS/MS-MRM

By MRM mode, quasi-molecular ions [Ganglioside-H]⁻/[Ganglioside-2H]²⁻ and the intensive fragment ions were selected to perform MS detection. The optimized response was performed by separately injecting a single ganglioside standard. Finally, 290.125 was determined as the quantitative daughter ion for all kinds of gangliosides, since sialic acid was easily detached from ganglioside. The more numbers of sialic acid in gangliosides were consistent with the smaller collision energy and RF lens voltage. In addition, the same series of ganglioside share the equal collision energy and RF lens voltage. The optimal product ions, collisional energy and RF lens voltage of 49 ganglioside species are determined and the results are shown in Table 1.

Different amounts of ganglioside standards were used to determine the linearity, the limit of detection (LOD) and limit of quantification (LOQ) for the targeted method. Calibration curves were drawn based on the concentration and peak area with favorable linearity. The LOD varies from 0.15 to 0.52 ng/mL, and LOQ varies from 0.5 to 1.72 ng/mL (Table 2).

Elevated serum GM3 as a potential biomarker in breast cancer diagnosis

Gangliosides extracts from serum samples of thirty female healthy adults, twenty benign breast tumor patients and seventy breast cancer patients were analyzed by LC-MS/MS-MRM method mentioned above. As the result showed, ganglioside levels in serum appear to be significantly higher in the cancer group than in the normal group

Table 1 Selected reaction monitoring conditions for 49 gangliosides by LC-MS/MS-MRM

Ganglioside	Compound	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)	
GD3[M-2H] ²⁻	GD3-34:2	Negative	720	290.125	28.90	140.00	
	GD3-34:1	Negative	721	290.125	28.90	140.00	
	GD3-36:1	Negative	735.1	290.125	28.90	140.00	
	GD3-38:1	Negative	749.1	290.125	28.90	140.00	
	GD3-40:2	Negative	762.1	290.125	28.90	140.00	
	GD3-40:1	Negative	763.1	290.125	28.90	140.00	
	GD3-41:1	Negative	770.1	290.125	28.90	140.00	
	GD3-42:3	Negative	775.1	290.125	28.90	140.00	
	GD3-42:2	Negative	776.1	290.125	28.90	140.00	
	GD3-42:1	Negative	777.1	290.125	28.90	140.00	
	GD1[M-2H] ²⁻	GD1-34:1	Negative	904	290.125	35.00	180.00
		GD1-36:2	Negative	917	290.125	35.00	180.00
GD1-36:1		Negative	918	290.125	35.00	180.00	
GD1-38:1		Negative	932	290.125	35.00	180.00	
GD1-42:2		Negative	959	290.125	35.00	180.00	
GT1b[M-2H] ²⁻	GT1b-34:1	Negative	1049.6	290.125	28.00	185.00	
	GT1b-36:1	Negative	1063.6	290.125	28.00	185.00	
GM3[M-H] ⁻	GM3-34:2	Negative	1149.8	290.125	44.79	249.00	
	GM3-34:1	Negative	1151.8	290.125	44.79	249.00	
	GM3-35:1	Negative	1165.8	290.125	44.79	249.00	
	GM3-36:3	Negative	1175.8	290.125	44.79	249.00	
	GM3-36:2	Negative	1177.8	290.125	44.79	249.00	
	GM3-36:1	Negative	1179.8	290.125	44.79	249.00	
	GM3-37:1	Negative	1193.8	290.125	44.79	249.00	
	GM3-38:3	Negative	1203.8	290.125	44.79	249.00	
	GM3-38:2	Negative	1205.8	290.125	44.79	249.00	
	GM3-38:1	Negative	1207.8	290.125	44.79	249.00	
	GM3-39:2	Negative	1219.8	290.125	44.79	249.00	
	GM3-39:1	Negative	1221.8	290.125	44.79	249.00	
	GM3-40:3	Negative	1231.8	290.125	44.79	249.00	
	GM3-40:2	Negative	1233.8	290.125	44.79	249.00	
	GM3-40:1	Negative	1235.8	290.125	44.79	249.00	
	GM3-41:2	Negative	1247.9	290.125	44.79	249.00	
	GM3-41:1	Negative	1249.9	290.125	44.79	249.00	
	GM3-42:4	Negative	1257.9	290.125	44.79	249.00	
	GM3-42:3	Negative	1259.9	290.125	44.79	249.00	
	GM3-42:2	Negative	1261.9	290.125	44.79	249.00	
	GM3-42:1	Negative	1263.9	290.125	44.79	249.00	
	GM3-43:2	Negative	1275.9	290.125	44.79	249.00	
	GM3-43:1	Negative	1277.9	290.125	44.79	249.00	
GM2[M-H] ⁻	GM2-34:1	Negative	1354.8	290.125	53.28	248.76	
	GM2-36:2	Negative	1380.9	290.125	53.28	248.76	
	GM2-36:1	Negative	1382.9	290.125	53.28	248.76	
GM1[M-H] ⁻	GM1-34:1	Negative	1516.9	290.125	55.00	248.76	
	GM1-36:1	Negative	1544.9	290.125	55.00	248.76	
	GM1-38:1	Negative	1572.9	290.125	55.00	248.76	
	GM1-40:2	Negative	1598.9	290.125	55.00	248.76	
	GM1-42:2	Negative	1626.9	290.125	55.00	248.76	
GM1-42:1	Negative	1628.9	290.125	55.00	248.76		

Fig. 1 The extracted peak area of gangliosides in serum samples by RP-HPLC-FTMS method.

Normal: healthy volunteers, $n = 10$; Cancer: breast cancer patients, $n = 20$. ($p < 0.05$, *; $p < 0.01$, **)

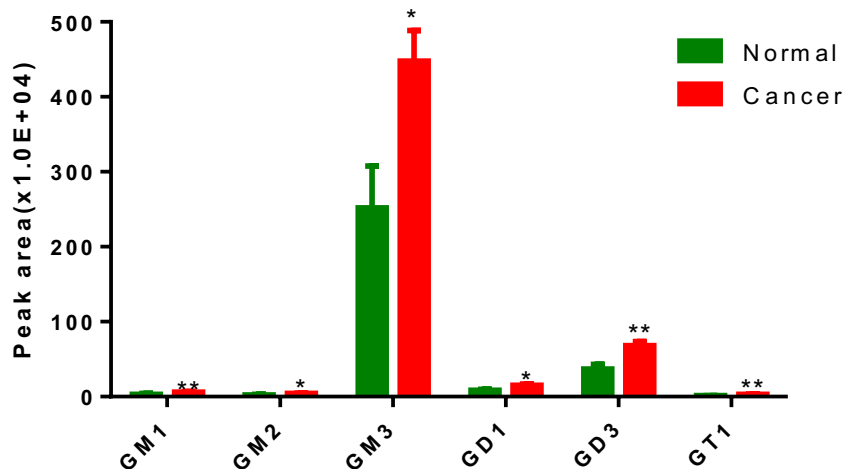


Table 2 Methodological evaluation of 6 ganglioside standards obtained by LC-MS/MS-MRM analysis

Analytes	$y = ax + b$			LOD ^a (p g / μL)	LOQ ^a (p g / μL)
	Slope <i>a</i>	Intercept <i>b</i>	R ²		
GM1–36:1	−0.50	8.20	0.9994	0.44	1.47
GM2–36:1	−0.21	4.78	0.9999	0.18	0.61
GM3–41:1	−0.32	3.18	0.9990	0.24	0.81
GD1–38:1	−0.04	3.47	1.0000	0.16	0.53
GD3–41:1	−0.04	4.56	0.9996	0.15	0.50
GT1–38:1	−0.02	1.91	1.0000	0.52	1.72

^a LOD, limit of detection (S/N = 3); LOQ, limit of quantification (S/N = 10)

(Fig. 2). The variation trend and relative abundance of gangliosides were accordant under both nontargeted and targeted methods. The average ganglioside levels in sera from breast cancer patients were 2~3 folds different than the healthy sera (Table 3). According to ROC curve analysis, GM1, GM2, GM3, GD1 and GD3 exhibited increased abundance in cancer serum compared with normal serum (AUC > 0.7, *p* value less than 0.01). In particular, as the major glycosphingolipid component, GM3 showed excellent diagnostic accuracy in cancer serum (AUC > 0.9) with further exploration value. In comparing the ganglioside profiles of healthy individuals with those suffering from breast cancer, seven ganglioside species with AUC greater than 0.9 indicated excellent diagnostic accuracy (Fig. 3), and all of them were GM3 species.

In order to obtain a general overview of GM3 profiles that could be characteristic for cancer conditions, PCA was conducted, which was a statistical procedure that extracting the dominant patterns in the matrix in terms of a complementary set of score and loading plots [36]. The acquired 23 GM3 profiles data, measured for normal and cancer subjects, were subjected to PCA as described

above to assess whether the global GM3 profile could separate cancer serum from normal. The PCA plotting result is shown in Fig. 4. Although complete separation between cancer and normal serum was not achieved, a clear distinction was observed, indicating changes in GM3 associated with adenocarcinoma.

In addition, the GM3 levels of cancer serum also differed from benign serum. The ROC curve revealed GM3 displayed better diagnostic value in sera of cancer patients compared with benign patients (AUC > 0.8) (Fig. 2 and Table 3), and four GM3 species exhibited better diagnostic accuracy (AUC > 0.8) (Fig. S1). However, the benign group couldn't be effectively differentiated with the other two groups through PCA analysis. Furthermore, in comparing with the normal group, GM3 showed evident increase in the benign group (AUC > 0.7) (Fig. 2 and Table 3), and two GM3 species exhibited better diagnostic accuracy (AUC > 0.7) (Fig. S2). On the basis of the AUC value and fold change, GM3 was identified as the best potential biomarker to distinct normal, benign and cancer group.

Elevated serum GM3 as a potential biomarker in luminal B subtype distinction

Compared with both normal and benign groups, total GM3, GM3–38:1, GM3–40:1 and GM3–40:2 all exhibited great diagnostic accuracy in breast cancer group in term of the results above (AUC > 0.8). The diagnostic value of GM3 was compelling, and relative diagnostic usefulness of GM3 on subtypes distinction were as follows.

The levels of gangliosides in different cancer subtypes also differed significantly. Compared with LB subtype, GM3 concentration in LA, HER and BS subtypes showed a significant decrease in patient serum (Fig. 5a). The ROC curve of gangliosides for different subtypes in Fig. 5b, d suggested GM3 as potential diagnostic marker to differentiate LB subtype from other subtypes.

Fig. 2 Gangliosides concentration in serum samples. Normal: healthy volunteers, *n* = 30; Benign: benign breast tumor patients, *n* = 12; Cancer: breast cancer patients, *n* = 70. (*p* < 0.05, *; *p* < 0.01, **)

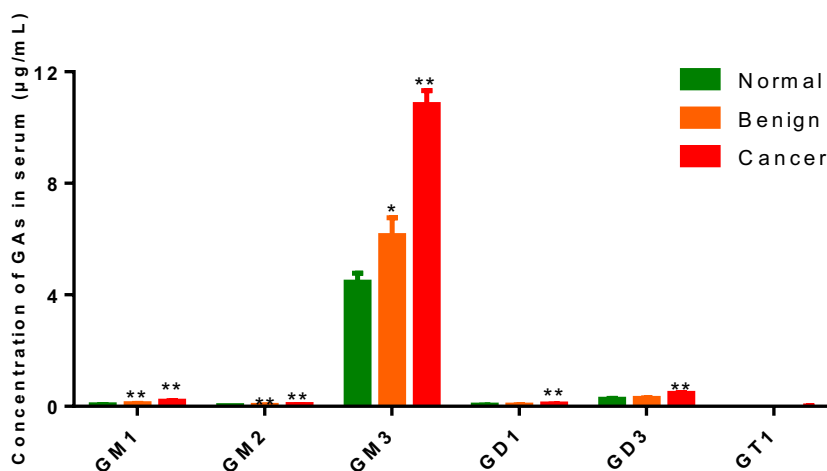
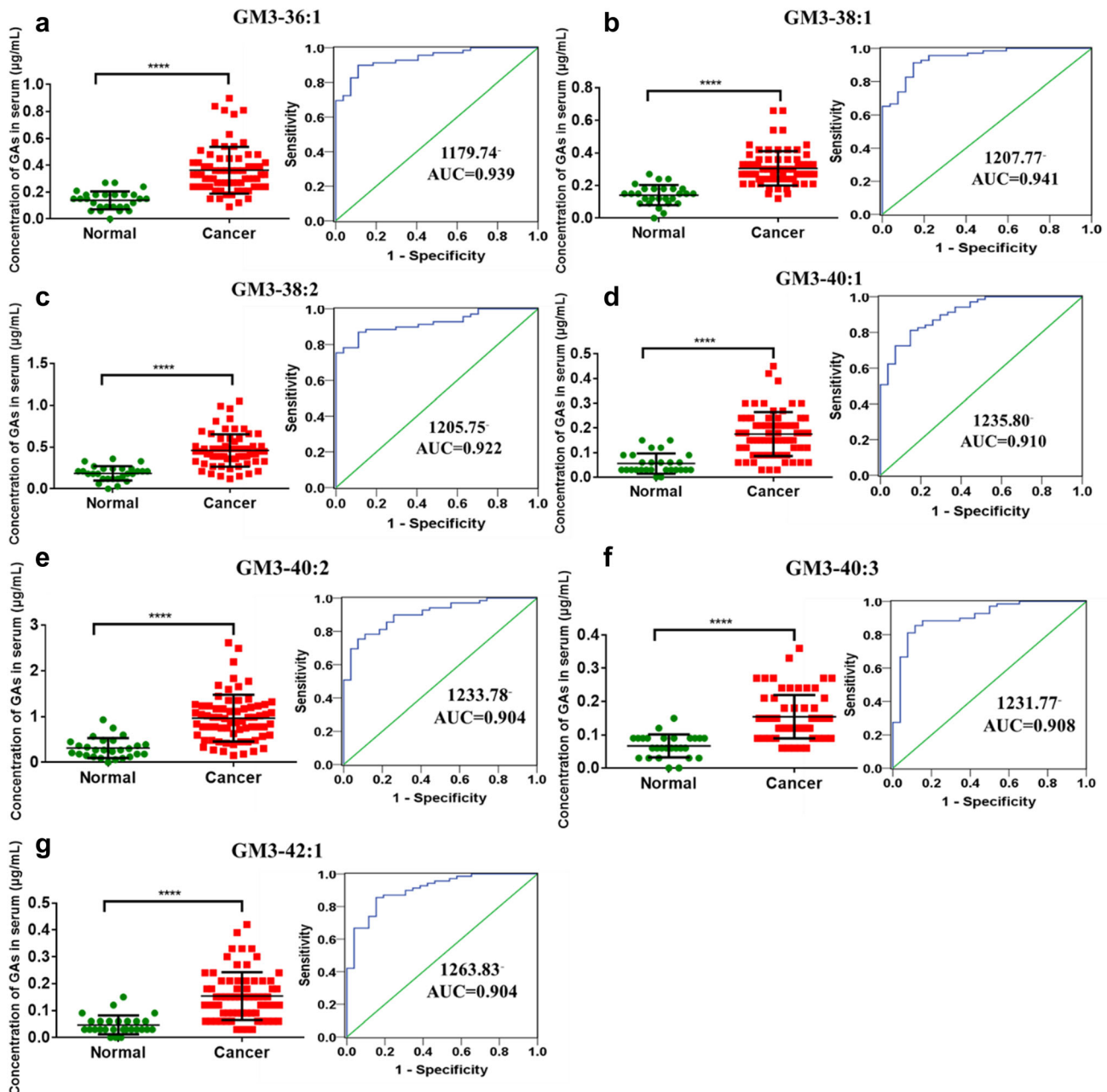


Table 3 Validation of the identified serum gangliosides from Normal, Benign and Cancer groups

	Benign vs Normal AUC/P value	Cancer vs Normal AUC/P value	Cancer vs Benign AUC/P value	Normal Mean ± SD (µg/mL)	Benign Mean ± SD (µg/mL)	Cancer Mean ± SD (µg/mL)	Benign/ Normal ratio	Cancer/ Normal ratio	Cancer/ Benign ratio
GM1	0.813/0.00	0.903/0.00	0.767/0.00	0.057 ± 0.007	0.099 ± 0.011	0.187 ± 0.013	1.68	3.30	1.97
GM2	0.737/0.01	0.799/0.00	0.658/0.03	0.024 ± 0.003	0.039 ± 0.005	0.062 ± 0.006	1.63	2.54	1.56
GM3	0.750/0.00	0.965/0.00	0.834/0.00	4.452 ± 0.327	6.127 ± 0.635	10.829 ± 0.500	1.38	2.43	1.77
GD1	0.651/0.08	0.730/0.00	0.685/0.01	0.042 ± 0.008	0.045 ± 0.005	0.081 ± 0.007	1.06	1.92	1.80
GD3	0.651/0.08	0.828/0.00	0.783/0.00	0.257 ± 0.029	0.282 ± 0.027	0.465 ± 0.025	1.10	1.81	1.64
GT1	0.615/0.19	0.576/0.25	0.323/0.02	0.003 ± 0.002	0.006 ± 0.002	0.009 ± 0.002	1.82	2.61	1.43

**Fig. 3** Ganglioside species which showed significant diagnostic accuracy (AUC > 0.9) in cancer serum (n = 70) compared with healthy serum (n = 30). The dot plot (left) and the ROC curve (right) of increased ganglioside concentration (a–g). ($p < 0.0001$, ****)

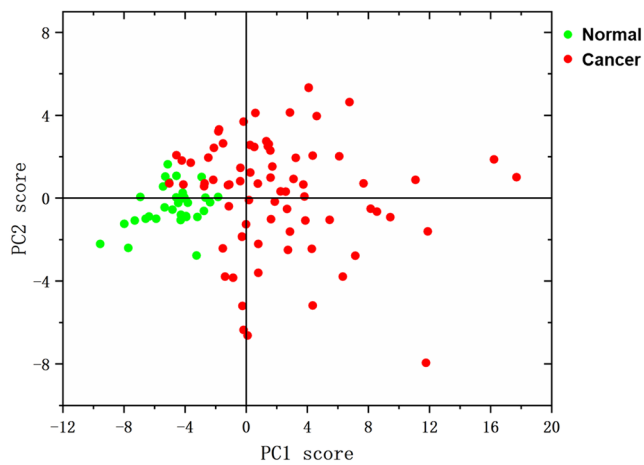


Fig. 4 PCA score plot of GM3 profiles derived from the serum of Normal (n = 30) and Cancer (n = 70)

Correlation between specific GM3 and clinical pathologic features

The Pearson Correlation for comparison of GM3 concentrations and age, ER, PR, the Her-2 receptor and Ki-67 status was analyzed in breast cancer patients. Twenty-three types of GM3 in the cancer group were

chosen for Pearson correlation analysis. As the results showed, a total of 17 GM3 types were correlated with the clinical pathologic characteristics of tumors, and most of them were related to Ki-67 status (Table 4). There was a positive correlation between GM3 and Ki-67 status. In addition, GM3–37:1 were positively related to age of patients. Four species of GM3 were positively correlated to ER status, while GM3–39:2 was correlated to PR status. GM3–38:1 and GM3–39:1 was positively related to HER-2 status.

Discussion

Lipidomics strategies have been divided into nontargeted and targeted approaches, each with their own advantages and disadvantages. Nontargeted lipidomics is the rapid screening of all the detectable analytes in large collections of biological samples, including chemical unknowns [37]. Conversely, targeted lipidomics is the measurement of defined groups of chemically characterized and biochemically annotated metabolites [38]. In the present study, LC-FTMS method was first used as a nontargeted method to identify all the ganglioside

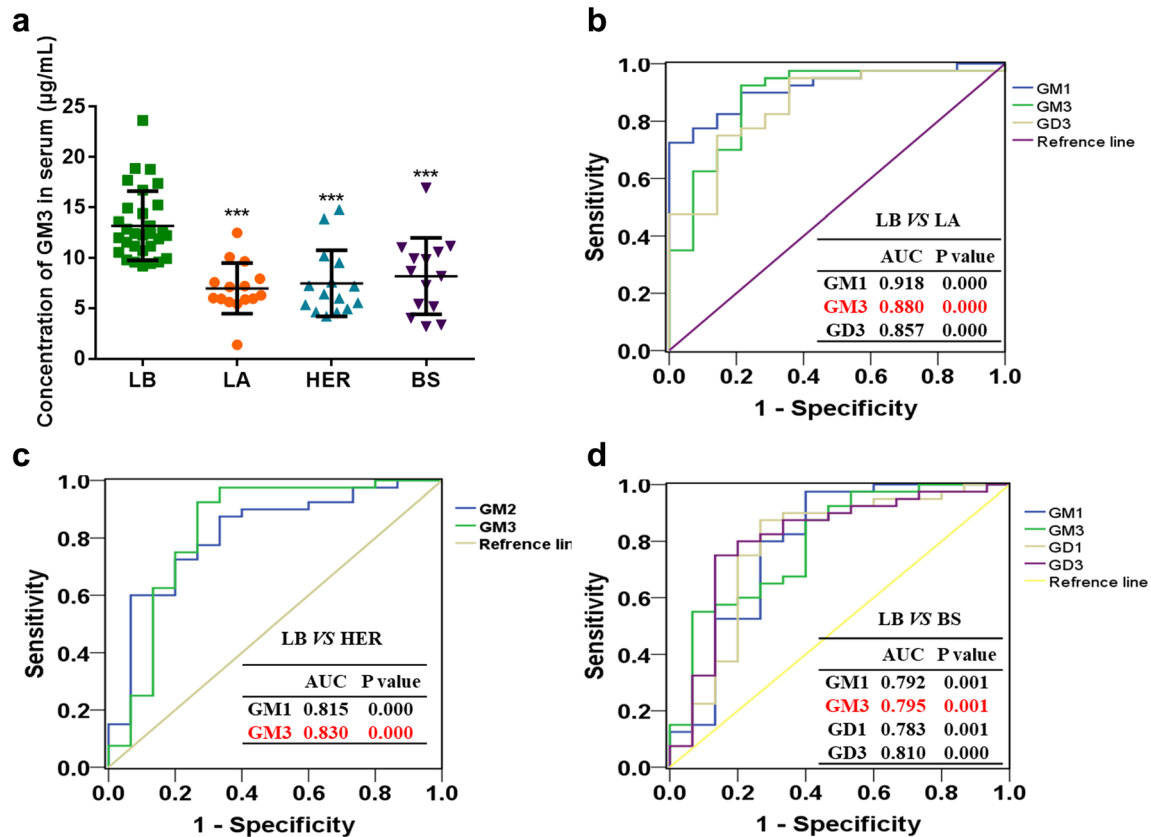


Fig. 5 GM3 concentration in patient serum of different subtypes (a) and the ROC curve of gangliosides which showed increased relative abundance for different subtypes: **b** LB subtype compared with LA subtype; **c** LB subtype compared with HER subtype; **d** LB subtype

compared with BS subtype. LA: Luminal A subtype, n = 13, LB: Luminal B subtype, n = 28, HER: HER-2 overexpressing subtype, n = 15, BS: basal-like subtype, n = 14. ($p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****)

Table 4 The pearson correlation coefficient between GM3 and clinical pathological features^a

Composition	Age	ER status	PR status	HER-2 status	Ki-67 status
GM3–34:1	–	–	–	–	0.254(0.029)
GM3–37:1	0.240(0.042)	–	–	–	–
GM3–38:1	–	–	–	0.284(0.014)	0.279(0.016)
GM3–38:2	–	0.250(0.032)	–	–	0.268(0.021)
GM3–39:1	–	–	–	0.238(0.041)	0.290(0.012)
GM3–39:2	–	0.262(0.024)	0.229(0.050)	–	0.273(0.019)
GM3–40:1	–	–	–	–	0.341(0.003)
GM3–40:2	–	0.242(0.038)	–	–	0.334(0.004)
GM3–40:3	–	–	–	–	0.287(0.013)
GM3–41:1	–	–	–	–	0.282(0.015)
GM3–41:2	–	0.245(0.036)	–	–	0.326(0.005)
GM3–42:1	–	–	–	–	0.337(0.003)
GM3–42:2	–	–	–	–	0.352(0.002)
GM3–42:3	–	–	–	–	0.318(0.006)
GM3–42:4	–	–	–	–	0.227(0.051)
GM3–43:1	–	–	–	–	0.274(0.018)
GM3–43:2	–	–	–	–	0.318(0.006)

^a The values were annotated by pearson correlation coefficient (*p* value)

species in serum, then LC-MS/MS-MRM method was employed as a targeted method to evaluate the levels of gangliosides in sera from healthy volunteers and patients with benign breast tumor or breast cancer before treatment. Eventually, 49 ganglioside species were determined, which included GM1, GM2, GM3, GD1, GD3 and GT1 species.

Significantly increases were observed in GM1, GM2, GM3, GD1 and GD3, which was consistent with the reported results that identified gangliosides from sera of breast cancer patients by 2D-HPTLC and GC-MS [39]. In particular, as the major glycosphingolipid component, GM3 showed excellent diagnostic accuracy in cancer serum (AUC > 0.9), which provided additional evidence that the level of GM3 was correlated with the occurrence of cancer. Several reports have shown GM3 being relevant to carcinogenesis. For example, inducible cell surface expression of GM3 was associated with reversion of malignancy through an integrin- and CD9-dependent mechanism [40, 41].

The vital roles that GM3 plays in various diseases has been widely reported. Elevated GM3 plasma concentration was identified in idiopathic Parkinson's disease [42]. A significant decrease of serum GM3 in early relapsing-remitting multiple sclerosis was observed and could be used as biomarkers of blood-brain barrier destruction [43]. Moreover, GM3 showed high levels at the late stage of melanoma metastasis [44]. GM3 synthase gene is a novel biomarker for histological classification in non-small cell lung cancer [45]. The pathological information of patients involved in our study was collected, and none of the patients suffered such disease that GM3 altered sharply. In our study, elevated serum GM3 was considered as potential biomarker in breast cancer diagnosis. However, the increase of GM3 expression in serum is not specific to breast

cancer, thus the diagnosis might be more accurate in combination with other clinical features.

Li *et al.* analyzed 512 lipid species in human plasma through 2D-LC/MS, and demonstrated clear separation of healthy people, benign breast tumor patients and breast cancer patients [46]. However, gangliosides were not taken into account in their paper, and our study indicated the abilities of gangliosides in diagnosis of breast cancer. Through PCA profile of GM3 species, a clear distinction was obtained between breast cancer patients and healthy volunteers. The ROC curve indicated diagnostic accuracy between cancer and benign sera, where the patient sera did not include other disorders where GM3 would be elevated. Ultimately, GM3 was identified as a potential biomarker to discriminate sera of breast cancer patients from healthy volunteers and benign breast tumor patients with high sensitivity.

In addition, GM3 was also considered as a diagnostic marker to differentiate LB subtype from other subtypes. Luminal B breast cancer, a subtype of ER-positive breast cancer, was defined by increased proliferation, poor outcome with endocrine therapy, and relative resistance to chemotherapy compared with other highly proliferative breast cancers [47]. Our study suggested the identification ability of GM3 on luminal B breast cancer and might guide clinical individualized treatment.

According to the previous report, Ki-67 was a useful marker of cell proliferation in early breast cancer, and Ki-67 was a prognostic parameter in breast cancer patients [48, 49]. Our results indicated a positive correlation between GM3 and Ki-67 status, suggesting the correlation of GM3 with cell proliferation and prognosis of breast cancer. What's more, it was noted that GM3 synthetase,

ST3GAL5, was correlated significantly to overall survival of breast cancer patients [50]. This highlights the need for further investigation of GM3 on breast cancer prognosis.

In this study, gangliosides obtained from breast cancer serum were analyzed by both nontargeted and targeted lipidomics method. LC-FTMS method was first used to measure all the ganglioside species in serum, then LC-MS/MS-MRM method was employed to evaluate the levels of gangliosides in serum from healthy volunteers and patients with benign breast tumor or breast cancer. Finally, GM3 was identified as a potential biomarker to discriminate sera of breast cancer patients from healthy volunteers and benign breast tumor patients. Additionally, GM3 was a potential diagnostic marker to differentiate LB subtype from other subtypes. A positive correlation between GM3 and Ki-67 status was identified. In conclusion, our results introduced the alteration patterns of serum gangliosides in breast cancer and suggested GM3 as a potential biomarker to diagnose breast cancer and distinguish LB subtype.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Abbreviations *LA*, Luminal A subtype; *LB*, Luminal B subtype; *HER*, HER-2 overexpressing subtype; *BS*, Basal-like subtype; *Normal*, Healthy volunteers; *Benign*, Benign breast tumor patients; *Cancer*, Breast cancer patients; *ROC*, Receiver operator characteristics; *PCA*, Principal component analysis; *MRM*, Multiple reaction monitoring; *LC-FTMS*, Liquid chromatography-Fourier transform mass spectrometry.

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