Primary Study of Lymph Node Metastasis-Related Serum Biomarkers in Breast Cancer

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ABSTRACT

The aim of this study was to detect the pretreatment serum protein profiles of breast cancer patients by mass spectrometry (MS) to screen candidate tumor biomarkers, which will supply a simple, accurate, and minimally invasive method to predict the axillary lymph node metastasis of breast cancer. We used magnetic bead-based weak cation-exchange chromatography followed by matrix-assisted laser desorption and ionization time-of-flight MS to detect proteins in the sera of 54 cases of axillary node-negative breast cancer, 47 cases of axillary node-positive breast cancer, and 101 healthy controls. The protein profiles were analyzed to screen tumor biomarkers and lymph node metastasis-associated proteins to establish and verify a diagnostic model. Comparison of the protein profiles between the two cancer groups resulted in a total of 111 discriminate *m/z* peaks that were associated with breast cancer. Furthermore, 40 discriminate *m/z* peaks were detected between breast cancer patients with and without axillary node metastases. Four protein *m/z* peaks at 5,643, 4,651, 2,377, and 2,240 were used to construct a diagnosis model, and cross-validation indicated that breast cancer with and without axillary node metastasis was identified with 87.04% sensitivity (47/54), 87.23% specificity (41/47), and 87.13% accuracy (88/101). These proteins could potentially be used as predictive biomarkers to distinguish between breast cancer patients with or without lymph node metastasis. Anat Rec, 294:1818–1824, 2011.

Key words: breast cancer; proteomics; diagnosis; lymph node; metastasis

Breast cancer is characterized by occult lymph node metastases and a high degree of malignancy and it is the second leading cause of cancer death among women next to lung cancer (Jemal et al., 2009). Lymph node metastasis is a common form of breast cancer and has been used as an independent prognostic factor (de Boer et al., 2010). Accordingly, improved methods for the diagnosis of lymph node metastases are needed for clinical staging and treatment guidance. The prevalent diagnostics used to identify lymph node metastasis include clinical palpation, imaging examination, lymph node biopsy, and sentinel node biopsy. Palpation diagnoses are subjective. The accuracy of palpation diagnoses depend on the examiner's experience, the size and location of the affected lymph node, and other factors. Prati et al. (2009) reported that the accuracy of lymph node

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metastasis diagnoses by clinical palpation was 65% (sensitivity 74%, specificity 56%). Chae et al. (2009) reported lymph node metastasis diagnoses using imaging diagnostics with 77.8%, 76.9%, and 73.2% accuracy and 51.5%, 33.3%, and 48.5% sensitivity by ultrasound, breast X-ray mammography, and positron emission tomography/computed tomography, respectively. Hsiang et al. (2007) reported that the accuracy of lymph node metastasis diagnoses by dynamic contrast-enhanced magnetic resonance imaging technology was 74% (sensitivity 62% and specificity 82%). In addition, axillary lymph node biopsy is usually blind. The use of ultrasound-guided fine-needle aspiration of lymph nodes by Baruah et al. (2010) resulted in a sensitivity of only 28.5%. Moreover, the needle puncture necessary for biopsy can cause local hematoma, and multiple punctures can cause significant pain to patients. Recently, sentinel lymph node biopsy has become a standard treatment model for the early diagnosis of breast cancer patients who are axillary lymph node negative (Lyman et al., 2005). However, this diagnostic method is not suitable for all patients, and the detection efficiency is highly associated with metastasis size and texture and the surgical skills of medical team. In addition, the false-negative rate in predicting axillary lymph node status was reported to be 9.5% (Krag et al., 2009). It is difficult to evaluate the axillary lymph node initial state, especially for neoadjuvant chemotherapy patients (Brown et al., 2010), and even for patients with positive sentinel lymph node biopsies, there are still more than 60% of patients with negative nonsentinel lymph nodes (Benson et al., 2007); thus, in these patients axillary lymph node biopsy or surgical resection would be unnecessary and not effective to improve the local disease control rate and long-term survival. Tamaki et al. (2009) showed that detection of cytokeratin 19 (cytokeratin 19 mRNA) can be used as a breast cancer marker in sentinel lymph node metastases with an accuracy reaching 92.9%; however, this method requires surgical excision of the lymph node tissue. Therefore, there is an urgent need to develop a safer and more effective forecasting system to predict axillary lymph node status, such that most axillary lymph node-negative patients can avoid surgery, thereby reducing suffering and medical expenses.

Proteomics can be a dynamic, systematic, and quantitative means to study changes in the type and quantity of proteins during the course of disease progression, enabling researchers to identify new drug targets and disease-specific biomarkers to improve diagnosis and prognosis. Mass spectrometry (MS) technology has been widely used in cancer research due to its small sample volume, simple operation, high sensitivity, and high-throughput capacity. This technique has accelerated clinical research and offers broader strategies for finding novel serum biomarkers (Petricoin et al., 2002; Engwegen et al., 2006; Garrisi et al., 2008; Zeidan et al., 2008; Li et al., 2009; Wang et al., 2009; Zeng et al., 2010). We designed experiments to analyze the serum protein profiles of 101 breast cancer patients and 101 healthy people using matrix-assisted laser desorption and ionization time-of-flight MS (MALDI-TOF-MS) to screen for candidate cancer biomarkers. Among the breast cancer patients, we compared serum protein profiles of the 54 patients with lymph node metastases and the 47 patients without lymph node metastases to screen for different proteins related to axillary lymph node metastasis. We sought to establish and verify a diagnostic model for the simple, rapid, accurate, and minimally invasive detection of lymph node metastasis in breast cancer patients.

PATIENTS AND METHODS

Patients

Experiments were performed at Zhejiang Cancer Hospital (Zhejiang, China) in January 2010. Serum samples were collected from patients diagnosed with breast cancer ($n = 101$) at Zhejiang Cancer Hospital, according to clinical criteria and further confirmed by histopathological analysis. Comparative studies were performed using serum samples from 101 healthy volunteers as controls. The study was approved by the local Ethics Committee of Zhejiang Cancer Hospital, and the patients and volunteers provided their informed consent. Serum samples were collected in 5 mL BD Vacutainers without anticoagulation and were allowed to clot at room temperature for up to 1 hr. The samples were then centrifuged at 4°C for 5 min at 10,000 rpm. The serum samples were frozen and stored at −80°C for future analysis.

Proteomic Analysis

Serum samples were pretreated with weak cation-exchange chromatography (WCX) magnetic beads (SED®) (SFDA permit No.20100230) (Huzhou SED Bio-Medical Science and Technology). Next, 10 μL of each serum sample was mixed with 20 μL of U9 solution (9 mol/L urea, 2% CHAPS) in a 0.5-mL microcentrifuge tube and incubated for 30 min at 4°C. Denatured serum samples were diluted with 370 μL of binding buffer (50 mmol/L sodium acetate, 1 mL/L Triton X-100, pH 4.0). At the same time, 50 μL of WCX magnetic beads were aliquoted into a Polymerase chain reaction (PCR) tube, which was then placed in a magnet separator for 1 min. The supernatant was then discarded carefully using a pipette, and the magnetic beads were washed twice with 100 μL of binding buffer. Next, 100 μL of the diluted serum sample was added to the activated magnetic beads, mixed, and incubated for 1 hr at 4°C, after which the beads were washed twice with 100 μL of binding buffer. The bound proteins were then eluted from the magnetic beads using 10 μL of 0.5% trifluoroacetic acid. Next, 5 μL of the eluted sample was diluted 1:2 in 5 μL of SPA (saturated solution of sinapinic acid in 50% acetonitrile with 0.5% trifluoroacetic acid). Two microliters of the mixture was aspirated and spotted onto steel arrays (Huzhou SED Bio-Medical Science and Technology). After air drying for approximately 5 min at room temperature, protein crystals on the chip were scanned by MALDI-TOF-MS (SFDA permit No. 20100230; Huzhou SED Bio-Medical Science and Technology) to determine the masses and intensities of all peaks on an *m/z* range of 1,000 to 50,000 Da. The reader was set up as follows: mass range (1,000–50,000 Da), optimized mass range (2,000–25,000 Da), laser intensity (273), and sensitivity (8). Mass calibration was performed using an all-in-one peptide reference standard containing vasopressin (1084.2 Da), somatostatin (1637.9 Da), bovine insulin β chain (3495.9 Da), recombinant human insulin (5807.6 Da), and hirudin (7033.6 Da; Huzhou SED Bio-Medical Science and Technology).

Statistical Methods

Biomarker Wizard software 3.1 (Ciphergen) was used to perform relative abundance tests for the different *m/z*

TABLE 1. Patient characteristics

Variable	Lymph node metastasis positive		Lymph node metastasis positive		*P* value
	N	(%)	*N*	(%)	
Age, years					0.418
≥50	34	(62.96)	25	(53.19)	
<50	20	(37.04)	22	(46.81)	
Tumor size e					0.002
≤2 cm	6	(11.11)	18	(38.30)	
2 cm∼5 cm	38	(70.37)	27	(57.45)	
≥5	10	(18.52)	2	(4.26)	
Histologic type-no. (96)					0.061
Infiltration ductal	53	(98.15)	40	(45.11)	
Ductal carcinoma in situ	0	(0.00)	4	(8.51)	
Invasive lobular carcinoma	1	(1.85)	1	(2.13)	
Mucoid carcinoma	0	(0.00)	2	(4.26)	
Estrogen-receptor status-no. (96)					0.425
Negative	24	(44.44)	21	(44.68)	
Low expression	5	(9.26)	3	(6.38)	
Medium expression	9	(16.67)	8	(17.02)	
High expression	16	(29.63)	12	(25.53)	
Unknown	0	(0.00)	3	(6.38)	
Progesterone-receptor status-no. (96)					0.033
Negative	30	(55.56)	25	(53.19)	
Low expression	6	(11.11)	6	(12.77)	
Medium expression	14	(25.93)	4	(8.51)	
High expression	4	(7.41)	9	(19.15)	
Unknown	0	(0.00)	3	(6.38)	
P53 status-no. (96)					0.392
Negative	20	(37.04)	21	(44.68)	
Low expression	16	(29.63)	11	(23.40)	
Medium expression	7	(12.96)	3	(6.38)	
High expression	10	(18.52)	8	(17.02)	
Unknown	1	(1.85)	4	(8.51)	
HER-2 status-no. (96)					0.395
Negative	7	(12.96)	5	(10.64)	
Low expression	7	(12.96)	8	(17.02)	
Medium expression	26	(48.15)	19	(40.43)	
High expression	13	(24.07)	10	(21.28)	
Unknown	1	(1.85)	5	(10.64)	
Type of surgery-no. (96)					0.637
Halsted radical mastectomy	3	(5.56)	3	(6.38)	
Auchinclass modified radical mastectomy	50	(92.59)	44	(93.62)	
Breast-conserving surgery	1	(1.85)	0	(0.00)	
Family history					0.100
Positive	6	(11.11)	11	(23.40)	
Negative	48	(88.89)	36	(76.60)	
Pathologic stage					0.000
I	0	(0.00)	10	(21.28)	
IIa	3	(5.56)	35	(74.47)	
IIb	29	(53.70)	2	(4.26)	
IIIa	14	(25.93)	0	(0.00)	
IIIb	0	(0.00)	0	(0.00)	
IIIc	8	(14.81)	0	(0.00)	
Histologic grade					0.493
I	4	(7.41)	2	(4.26)	
II	27	(50.00)	27	(57.45)	
III	7	(12.96)	9	(19.15)	
Unknown	16	(29.63)	9	(19.15)	

proteins. *P* values of <0.05 were considered statistically significant. The data group and correlation were analyzed by Biomarker Pattern software 5.0 (Ciphergen). The counted data were compared by chi-squared test and Fisher exact test, and measurement data were analyzed by paired *t*-test; these analyses were performed using SPSS 7.0 statistical analysis software.

RESULTS

Clinical Characteristics of Breast Cancer Patients

Among the 101 breast cancer patients, the average age was 51.95 years (33–72 years), and 17 of these patients had a family history of breast cancer. There were 93 cases

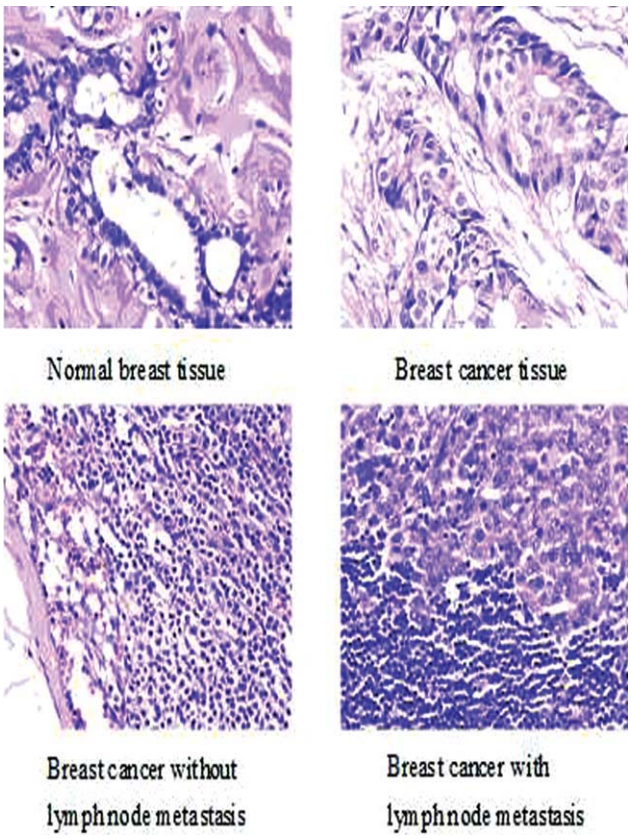

Fig. 1. Breast cancer and lymph node status.

of invasive ductal carcinoma, two cases of invasive lobular carcinoma, four cases of ductal carcinoma in situ, and two cases of mucinous carcinoma. For treatment, 94 patients received modified radical mastectomies, six patients received radical mastectomies, and one patient received a breast-conserving surgery. Clinical staging was assessed according to the 6th edition The American Joint Committee on Cancer (AJCC) breast cancer TNM (a cancer staging system to describe the extent of cancer) pathological staging standard: 10 cases were stage I, 69 cases were stage II, and 22 cases were stage III. Pathological examination postsurgery revealed that 54 of the 101 breast cancer patients had lymph node metastases, while 47 patients did not. The primary tumor size, progesterone receptor state, and pathologic staging of these two groups were different ($P < 0.05$). Specific clinical information can be seen in Table 1. Breast cancer and lymph node status are shown in Fig. 1.

Protein Profile Comparison between the Breast Cancer Group and Healthy Control Group

Normalized protein profiles of 101 breast cancer sera and 101 age-matched control sera were analyzed by Biomarker Wizard software, yielding 170 protein peaks between 1,000 and 50,000 Da. Of these, 111 protein peaks exhibited a significant difference between breast cancer and healthy control samples ($P < 0.05$), 83 of which exhibited a very significant difference ($P < 0.001$). Further analysis of these differentially expressed proteins by Biomarker Patterns software established *m/z* ratios of 3979, 5643, 6437, and 8929, and the model I (Molecular weight, mean values, and *P* values of these different peaks, see Table 2 and Fig. 2) was used to draw a map of the tree node (Fig. 3). Analysis of the MS results by this method led to 97 correct cases and four incorrect cases which were verified by biopsy, resulting in a sensitivity of 96.4% (97/101). Furthermore, 89 cases were correctly distinguished while 12 cases were incorrectly distinguished, resulting in specificity of 88.12% (89/101). Taken together, the accuracy of this method was 92.08% (186/202). According to the different proteins and their specific combinations, this method could theoretically distinguish breast cancer from healthy controls.

TABLE 2. Differentially expressed proteins in serum from breast cancer patients and normal individuals

Mass–charge ratio (*m/z*)	Average intensity of protein peak		*P* value
	Breast cancer	Normal	
3979.45	6.63 ± 5.51	2.91 ± 1.48	0
8929.60	12.98 ± 15.35	3.27 ± 1.75	0
5643.50	4.44 ± 2.84	2.36 ± 1.35	4.67E−08
6437.17	11.84 ± 5.78	8.65 ± 5.39	1.9559E−06

Comparing the Serum Protein Profiles of Breast Cancer Patients With and Without Lymph Node Metastasis

Protein *m/z* data of 54 breast cancer cases with lymph node metastasis and 47 cases without lymph node metastasis were compared and analyzed using Biomarker Wizard software, yielding 109 protein peaks in the range of 1,000–50,000 Da, 40 of which exhibited significant differences ($P < 0.05$), and 13 of which had very significant differences ($P < 0.001$). These differentially expressed serum proteins were further analyzed using Biomarker Patterns software, establishing *m/z* ratios of 4651, 5643, 2240, and 2377, and the four proteins diagnosis model II (molecular weights, mean values, and *P* values of different peaks shown in Table 3 and Fig. 4) was used to draw the map of the tree node (Fig. 5). Analyzing the MALDI-TOF-MS results of the 54 cases with lymph node metastasis revealed 47 correct cases and seven incorrect cases, resulting in sensitivity of 87.04% (47/54). Of the 47 cases without lymph node metastasis, 41 cases were distinguished correctly, and six were not, resulting in specificity of 87.23% (41/47). Overall, the accuracy of this diagnosis model was 87.13% (88/101). Theoretically, the diagnosis model II can distinguish breast cancer patients with or without lymph node metastasis.

DISCUSSION

Early diagnosis and early treatment are important to improve survival rates of breast cancer patients; unfortunately, currently used serum biomarkers of breast cancer, such as CA15.3, CA27.29, and CEA, exhibit low sensitivity and specificity (Harris et al., 2007). The rapid development of proteomic technologies provides reliable tools for screening distinct proteins in breast cancer patients. In this study,

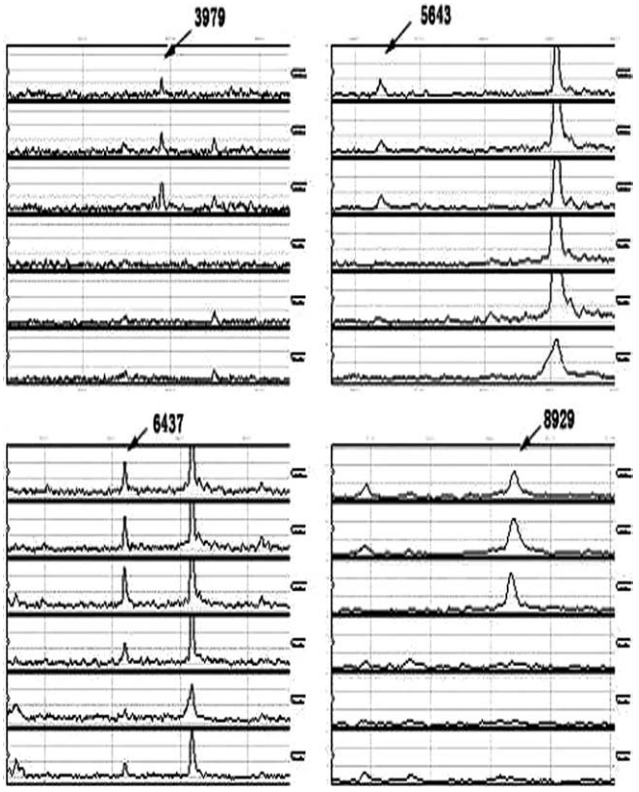

Fig. 2. The spectral views of selected biomarkers. Top three graphs: cancer; bottom three graphs: normal.

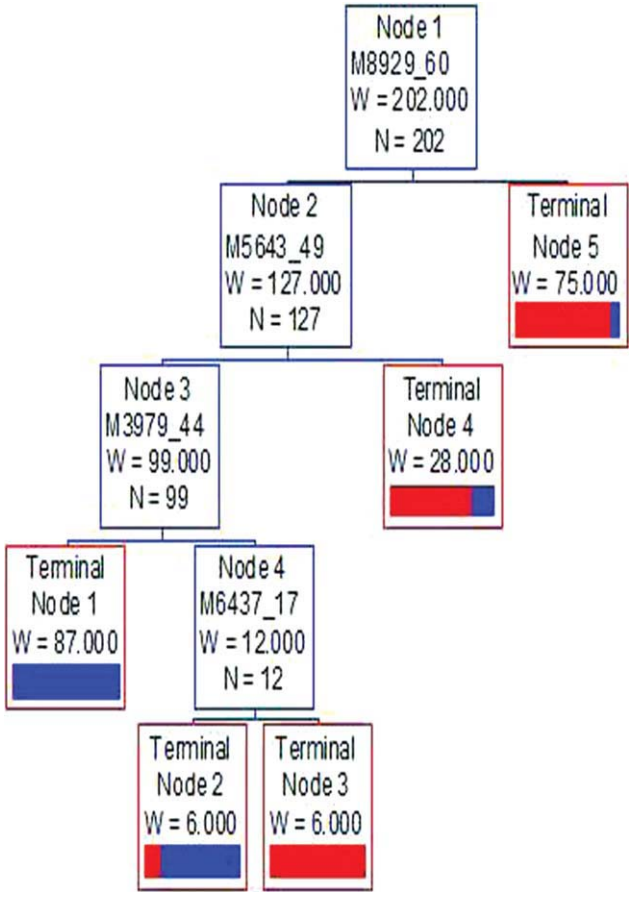

Fig. 3. Biomarker pattern classification tree model diagram. *m/z* 8,929 as the node 1, *m/z* 5,643 as the node 2, *m/z* 3,979 as the node 3, and *m/z* 6,437 as the node 4.

we found 111 protein peaks with significant differences between the breast cancer group and the control group using MALDI-TOF-MS and a proteomics approach. We then constructed an early diagnosis model with four proteins wherein the diagnosis accuracy of breast cancer reached 92.08% (186/202) with higher sensitivity and specificity than cancer biomarkers currently used in the clinic. Previous similar studies have shown (Mathelin et al., 2006; Belluco et al., 2007) that combining multiple protein peaks should result in greater sensitivity and specificity in the early diagnosis of breast cancer. Our study used different types of reagents and a bioinformatics approach, which may be useful to predict breast cancer among Chinese populations.

Axillary lymph node metastasis plays an important role in the preoperative evaluation, accurate staging, treatment guidance, and prognosis of patients with breast cancer. Axillary lymph node metastasis is a complex process involving multiple steps and multiple genes, including changes in tumor cell adhesion, migration, and proliferation. This process is regulated by genes and executed by functional proteins. The study of lymph node metastasis-relevant proteins provides clues about the metastasis mechanism, potential biomarkers for metastasis diagnosis, the prognosis of patients with an objective evaluation, and anti-metastasis drug targets, and it may prevent overtreatment or inadequate treatment. Currently, the use of proteomics research on tumor metastasis in prostate cancer (Pang et al., 2010), colon cancer (Xue et al., 2010), multiple myeloma (Wang et al., 2009) and larynx cancer (Cheng et al., 2008) have been successfully reported, but few studies have been reported in axillary lymph node metastasis of breast cancer. Nakagawa et al. (2006) reported the use of Surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI)-TOF-MS combined with microdissection to detect 65 cases of primary breast cancer and found that protein *m/z* peaks at 4871 and 8596 Da were associated with axillary lymph node metastasis. Hu and colleagues used two-dimensional liquid chromatography tandem MS (2D-LC-MS/MS) to detect seven lymph node-positive cases and seven lymph node-negative cases in breast cancer patients. They screened

TABLE 3. Differentially expressed proteins in serum of lymph node metastasis positive group and negative group

Mass–charge ratio (*m/z*)	Average intensity of protein peak		*P* value
	Positive group	Negative group	
5643.49	3.22 ± 2.16	5.84 ± 2.91	0.000002225
4651.31	9.28 ± 3.90	11.28 ± 3.00	0.000732907
2377.50	6.19 ± 2.25	4.43 ± 2.64	0.000959737
2240.93	9.35 ± 2.91	7.40 ± 3.14	0.001510223

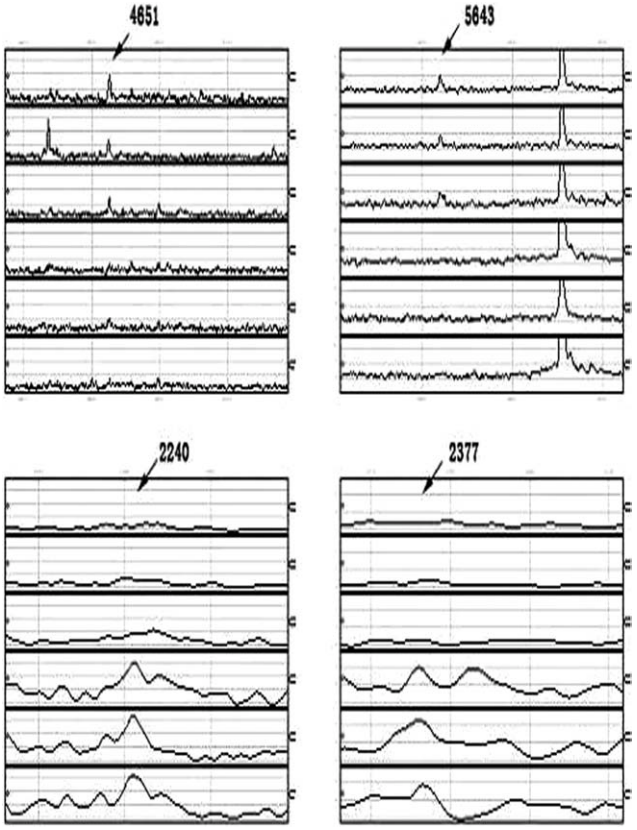

Fig. 4. Spectral views of selected biomarkers. Top three graphs: lymph node-negative; bottom three graphs: lymph node-positive.

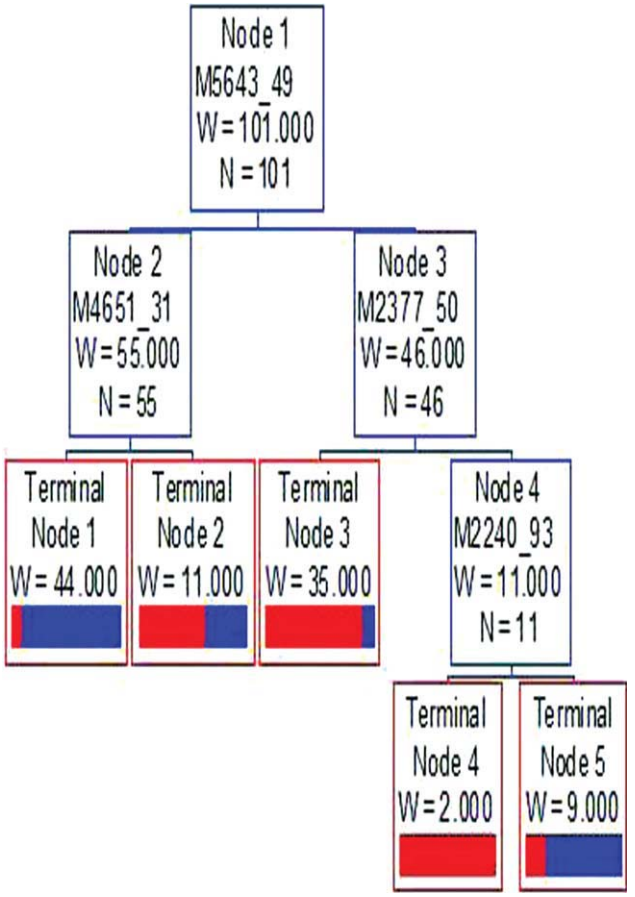

Fig. 5. Biomarker pattern classification tree model diagram. *m/z* 5,643 as node 1, *m/z* 4,651 as node 2, *m/z* 2,377 as node 3, and *m/z* 2,240 as node 4.

131 distinct proteins and verified tenascin-XB by Enzyme-linked immunosorbent assay (ELISA) as a diagnostic biomarker of lymph node metastasis (Hu et al., 2009). In this study, we compared serum protein profiles from 54 lymph node-positive cases and 47 lymph node-negative cases of breast cancer and found 40 protein peaks significantly related to axillary lymph node metastasis. We then compared our MS results with the results of axillary lymph node dissection to verify their predictive value. Based on our experimental results, the diagnostic model II can distinguish patients with or without lymph node metastasis; the diagnostic accuracy reached 87.13% (sensitivity 87.04% and specificity 87.23%). More studies on protein *m/z* peaks at 45643.49, 4651.31, 2377.50, and 2240.93 are under investigation. We intend to identify relevant biomarkers related to lymph node metastasis of breast cancer to predict metastases.

Proteomics, with unique methods and techniques, has been used to explore various facets of breast cancer; however, it is an extremely complex and heterogeneous disease that cannot be summarized using a simple model due to its multistage evolution process. In our study, we found novel candidate biomarkers and compared the *m/z* to the biomarkers identified in previous studies in breast cancer (Zeidan et al., 2008; Leong et al., 2007). The novel biomarkers could be detected in our study is due to using different mass spectrometry methods compared to the previous study (Villanueva et al., 2005). In this study, we adopted a strict standardization of methods, standard operating procedures, and an improved data analysis platform to reduce detection errors generated from technical experimental factors. In addition, we considered the age, genetics, tumor type, tumor grade, and other clinical factors that could result in experimental errors. We are optimizing the diagnostic model used and other factors to acquire more accurate protein identification, including optimizing the diagnostic model, expanding the sample size, and a follow-up, double-blind study to evaluate early diagnosis of breast cancer and prediction of axillary lymph node metastasis. To identify biomarkers that can predict things like metastasis size and survival are under further investigation.

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