Oncotype DX 21-gene test has a low recurrence score in both pure and mixed mucinous breast carcinoma

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Abstract. The Oncotype DX 21-gene test can be used to predict chemotherapy efficacy in patients with estrogen receptor (ER)-positive and HER2-negative breast cancer; however, the data on the 21-gene recurrence score (RS) for mucinous breast carcinoma (MBC) are limited. The present study aimed to evaluate the distribution pattern and clinical value of the 21-gene RS in patients with MBC. A total of 38 pure MBC (PMBC) and 11 mixed MBC (MMBC) cases were retrospectively analyzed, and a total of 29 ER-positive and HER2-negative MBCs underwent the Oncotype DX 21-gene test. There were no statistically significant differences between the PMBCs and MMBCs in age, tumor size and molecular subtype; however, patients with MMBC showed a significantly higher incidence rate of nodal metastases compared with that in patients with PMBC (72.7 vs. 16.2%, respectively). Following surgery, 87.8 and 59.2% of the enrolled patients received endocrine therapy and chemotherapy, respectively. With a median follow-up of 65.6 months, the 5-year disease-free survival and overall survival rates were 97.0 and 100.0%, respectively. The 21-gene test revealed that the proportions of patients with MBC categorized into low (RS <18), intermediate (RS ≥18-30) and high (RS ≥30) risk groups were 51.7, 44.8 and 3.5%, respectively, and there was no statistically significant difference between the PMBC and MMBC cases. Notably, among the genes in the 21-gene RS testing, the expression levels of cathepsin V, progesterone receptor (PR) and CD68 were significantly higher in the PMBC group compared with that in the MMBC group. In conclusion, the current study demonstrated that patients with MBC had a favorable prognosis, and both PMBC and MMBC cases had a low- and intermediate-risk RS, which suggests that a considerable proportion of patients may be able to avoid chemotherapy. In addition, the high expression level of PR, based on the 21-gene test in PMBCs, indicated that they may have a more favorable response to endocrine therapy than MMBCs.

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Introduction

Mucinous breast carcinoma (MBC) is a rare variant of breast cancer accounting for 1-6% of all primary breast carcinomas, and is characterized by small clusters of tumor cells floating in lakes of partitioned mucin (1,2). MBC has a more favorable prognosis compared with non-specific invasive ductal carcinoma (IDC), as most cases are associated with a high expression of estrogen and/or progesterone receptors (ER/PR$^+$) and a low expression of HER2 (3,4). In addition, most studies have reported that MBCs have a lower frequency of axillary lymph node metastases compared with IDCs (5), which also suggests that the treatment of MBC should be different from IDC, and additional detection methods should be used to guide the treatment of MBC. According to the tumor components, MBCs are divided into two subtypes: Pure MBC (PMBC), which is defined as a tumor with a mucinous component of >90%, and mixed MBC (MMBC), which is defined as a tumor with a 51-90% mucinous component and admixing, usually with an infiltrating ductal epithelial component (6,7). A previous study reported a difference in prognosis for PMBCs and MMBCs, with a lower frequency of axillary lymph node metastases and a more favorable outcome in the former subtype (8). However, whether the treatment of these two types of breast cancer should be differentiated remains unknown.

The Oncotype DX 21-gene recurrence score (RS) assay is calculated based on the results of a reverse transcription (RT)-PCR assay of 21 prospectively selected genes in tumor tissues (9). Over the past decade, the 21-gene RS has been widely used by clinicians to assist with predicting the outcomes and guides therapeutic decisions in patients with ER-positive/HER2-negative breast cancer, and it has become the only genomic test recommended by National

Comprehensive Cancer Network guidelines (10,11). Further validation studies also confirmed its ability to predict the benefit from chemotherapy (CT) both in node-negative and node-positive cases (12,13). Notably, the majority of MBCs have favorable features, including being ER-positive and HER2-negative and having a lower incidence rate of nodal metastasis, which matches the criteria of the 21-gene genomic test (14), thereby suggesting that a considerable number of patients with MBC may avoid unnecessary CT after the Oncotype DX 21-gene test. However, at present, data on the RS of MBC remains limited, due to its relative rarity, and it remains unknown whether the accuracy, practicability and effectiveness of the 21-gene RS test in guiding the treatment of IDC is also suitable for MBC due to tumor heterogeneity (15).

The present study retrospectively investigated the clinicopathological features and treatment patterns of 49 cases of MBC, and the Oncotype DX 21-gene RS test was performed in 29 cases of MBC. We hypothesized that the results of the 21-gene test could be used to guide the treatment in patients with MBC. Furthermore, the clinicopathological features and the 21-gene RSs were compared between patients with PMBC and those with MMBC. In addition, the individual gene expression from the 21-gene test was also analyzed between the PMBC and MMBC groups.

Materials and methods

Patients and follow-up. In total, 50 women who were diagnosed with MBC and treated at the Department of Thyroid and Breast Surgery, Affiliated Hospital of Zunyi Medical University (Guizhou, China) between February 2010 and February 2021, were retrospectively included. During this period, a total of 3,081 patients were diagnosed with breast cancer, and MBC accounted for 1.59%. The main inclusion criteria were as follows: Female, without distant metastasis at first diagnosis and confirmed to be MBC by the Pathology Department of The Affiliated Hospital of Zunyi Medical University. The main exclusion criteria were as follows: Male, bilateral cancer, presence of distant metastasis and unavailability of tissue samples. A total of 49 patients were included in this study according to the aforementioned criteria, and 1 patient was excluded due to the inability to obtain tissue samples. All available clinicopathological data, including age, menstrual status, tumor size, lymph node status, TNM stage, immunohistochemistry (IHC) results and treatment were collected from the medical records. The patients received all therapeutic procedures, such as surgery, adjuvant CT, irradiation and hormonotherapy at the same institution (Table I).

The time to follow-up was from the date of surgery to the date of recent follow-up. Patient follow-up was accomplished by specialized staff at the Department of Thyroid and Breast Surgery, Affiliated Hospital of Zunyi Medical University, and routine correspondence and telephone calls were used for follow-up. The follow-ups were performed every 3 months during the first 2 years, every 6 months during the next 3 years, then once a year thereafter. Overall survival (OS) time was calculated from the date of surgery to the occurrence death of any cause. Disease-free survival (DFS) time was estimated from the date of surgery until the date of first proven recurrence, including local/regional recurrence and distant metastasis at any site. The last follow-up was conducted in April 2021. The current study was approved by the Ethical Committee of The Affiliated Hospital of Zunyi Medical University. All procedures were in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was provided by all the patients, and all tissue samples used were from paraffin embedded tissues following surgery. The tumor tissue was fixed with 10% neutral buffered formalin at room temperature overnight, and the 4-μm thick tissue sections were used for pathological evaluation.

Hematoxylin & eosin (H&E) staining. H&E-stained slides of the MBCs were reviewed according to the 2012 World Health Organization classification criteria (16). The histological sections were stained with hematoxylin for 8-10 min and eosin for 4-5 sec at room temperature, then the stained sections were observed under a light microscope (magnifications x40 and x100). PMBCs were defined as having a mucinous component of >90% and MMBC was defined with a 51-90% mucinous component. In addition, hypocellular MBC (type A) and hypercellular MBC (type B) were also determined based on cell cluster density (17).

IHC analyses. ER, PR, HER-2 status and the Ki-67 index were evaluated using IHC. Briefly, the 4-μm thick tissue sections were incubated with the immunohistochemical antigen repair buffer (cat. no. MVS-0099; Beijing Strong Biotechnologies, Inc.) for 20 min after dewaxing in xylene for 60 min and rehydrated in a descending alcohol series (100, 95 and 75%) at room temperature. Subsequently, the tissue sections were blocked using an endogenous biotin blocking kit (cat. no. BLK-0002; Beijing Strong Biotechnologies, Inc.) for 10 min at room temperature. After washing with PBS, the tissue sections were incubated for 32 min at 42˚C with primary antibodies targeted against ER (cat. no. kit-0012; clone SP1; 1:100; rabbit monoclonal), PR (cat. no. kit-0013; clone SP2; 1:100; rabbit monoclonal), HER2 (cat. no. Kit-0043; clone MXR001; 1:100; rabbit monoclonal) and Ki-67 (cat. no. RMA-0731; clone MXR002; 1:100; rabbit monoclonal) (all from Beijing Strong Biotechnologies, Inc.). After washing with PBS, the tissue sections were processed with a Maxvision™ HRP kit (cat. no. kit-5004; Beijing Strong Biotechnologies, Inc.). The IHC results were judged by experienced pathologists using a light microscope (magnification, x40 and x100), and the ER and PR were regarded as positive if >1% of nuclei were stained (18). With respect to Ki-67, a range of 500-1,000 cells were counted to calculate the percentage of positive tumor cell nuclei, including hot spot areas (19). The molecular subtype was classified according to the 2013 St. Gallen expert panel consensus (20). All histological and IHC tumor slides were evaluated independently by two pathologists.

Fluorescence in situ hybridization (FISH). HER2 status was considered to be positive if >10% of the tumor cells showed a score of 3+ from IHC or showed a >2.2-fold increase in FISH using a HER2 DNA Probe kit (cat. no. 2J01-30; Abbott Molecular Inc.) (21). Briefly, after the samples were deparaffinized, dehydrated and air-dried, the tissue sections were handled with pre-treatment solution at 80˚C for 30 min.

Table I. Detailed IHC and adjuvant treatment information of the enrolled patients.

Case ID	ER status	PR status	HER2 status[a]	Ki67, %	Molecular subtype	Chemotherapy	Endocrine therapy	Irradiation
MMBC1	Positive	Negative	Negative	30	B	Yes	Yes	Yes
MMBC2	Positive	Negative	Negative	2	B	Yes	Yes	No
MMBC3	Positive	Negative	Negative	30	B	Yes	Yes	Yes
MMBC4	Positive	Positive	Negative	10	A	No	Yes	No
MMBC5	Positive	Positive	Negative	10	A	Yes	Yes	No
MMBC6	Positive	Positive	Negative	10	A	Yes	Yes	No
MMBC7	Positive	Positive	Negative	5	A	Yes	Yes	No
MMBC8	Positive	Positive	Positive	20	B/HER2	Yes	Yes	No
MMBC9	Positive	Negative	Negative	20	B	No	Yes	No
MMBC10	Positive	Positive	Positive	10	B/HER2	Yes	Yes	No
MMBC11	Positive	Positive	Positive	20	B/HER2	Yes	Yes	Yes
PMBC1	Positive	Positive	Negative	5	A	No	Yes	No
PMBC2	Positive	Positive	Negative	10	A	Yes	Yes	No
PMBC3	Positive	Positive	Negative	15	B	Yes	Yes	No
PMBC4	Positive	Positive	Negative	20	B	Yes	Yes	No
PMBC5	Positive	Positive	Negative	10	A	No	Yes	No
PMBC6	Positive	Positive	Negative	20	B	Yes	Yes	Yes
PMBC7	Positive	Positive	Negative	10	A	No	Yes	No
PMBC8	Positive	Positive	Negative	15	A	No	Yes	No
PMBC9	Positive	Positive	Negative	3	B	Yes	Yes	No
PMBC10	Positive	Positive	Negative	20	B	No	No	No
PMBC11	Positive	Positive	Negative	10	B	No	Yes	No
PMBC12	Positive	Positive	Negative	10	A	No	Yes	No
PMBC13	Negative	Negative	Negative	80	TNBC	No	No	No
PMBC14	Positive	Positive	Negative	20	B	Yes	Yes	No
PMBC15	Positive	Positive	Negative	10	A	Yes	Yes	No
PMBC16	Positive	Positive	Negative	5	A	No	Yes	No
PMBC17	Positive	Negative	Negative	5	B	Yes	Yes	No
PMBC18	Positive	Positive	Negative	60	B	Yes	Yes	Yes
PMBC19	Negative	Negative	Positive	20	HER2	Yes	No	No
PMBC20	Negative	Negative	Positive	40	HER2	Yes	No	Yes
PMBC21	Positive	Positive	Negative	15	A	Yes	Yes	No
PMBC22	Positive	Negative	Negative	10	B	No	Yes	No
PMBC23	Positive	Positive	Negative	5	A	No	Yes	Yes
PMBC24	Positive	Positive	Negative	10	A	Yes	Yes	No
PMBC25	Positive	Positive	Negative	10	A	No	Yes	No
PMBC26	Positive	Positive	Negative	10	A	Yes	Yes	Yes
PMBC27	Positive	Positive	Negative	5	A	No	Yes	No
PMBC28	Positive	Positive	Negative	10	A	No	Yes	No
PMBC29	Positive	Positive	Negative	20	B	No	Yes	No
PMBC30	Positive	Positive	Negative	10	A	No	Yes	Yes
PMBC31	Positive	Positive	Negative	50	B	Yes	Yes	No
PMBC32	Positive	Negative	Negative	20	B	Yes	Yes	Yes
PMBC33	Positive	Positive	Negative	40	B	Yes	Yes	Yes
PMBC34	Positive	Positive	Negative	10	A	Yes	Yes	No
PMBC35	Positive	Positive	Negative	1	A	No	Yes	No
PMBC36	Positive	Positive	Negative	10	A	Yes	Yes	No
PMBC37	Positive	Positive	Negative	10	A	No	Yes	No
PMBC38	Negative	Negative	Negative	5	TNBC	No	No	No

[a]From IHC and FISH. PMBC, pure mucinous breast carcinoma; MMBC, mixed mucinous breast carcinoma; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; A, luminal A subtype; B, luminal B subtype; HER2, human epidermal growth factor receptor 2 subtype; TN, triple-negative subtype.

Then, the sections were immersed in protease solution at 37˚C for 34 min, followed by immersion in wash buffer (70, 80 and 100% ethanol). Subsequently, the tissue sections were incubated with the probe mixture [10 μl HER2 probe (226 kb; 10 ng/μl) and 10 μl CEP17 probe (9 kb; 20 ng/μl)] at 74˚C for 5 min, then the cover slip was sealed with Fixogum rubber cement (cat. no. 12101ES62; Marabu GmbH and Co. KG) for 10 min at room temperature, and the samples were subsequently incubated overnight at 37˚C. Next, the samples were washed with post-hybridization wash buffer at room temperature for 15 min. After air-drying, 10 μl DAPI (cat. no. 30-804840; Abbott Molecular Inc.) was added to the target area and a cover glass was added and the samples were incubated at -20˚C for 10 min. After the slides were stored in the dark and left at room temperature, the FISH results were judged by experienced pathologists using a fluorescence microscope (magnification, x40 and x100).

Testing using the 21-gene RS assay. The Oncotype DX 21-gene test was performed by AmoyDx Diagnostics Co., Ltd. Briefly, the H&E-stained slides were reviewed by pathologists to ensure that the paraffin section contained sufficient tumor tissue. RNA was then extracted from the unstained breast tumor formalin fixed paraffin-embedded (FFPE) sections using a RNeasy FFPE RNA kit (cat. no. 172348; AmoyDx Diagnostics Co., Ltd), and the concentration was measured after verifying the absence of DNA contamination. Gene-specific RT was performed at 65˚C for 5 min and 37˚C for 60 min using the PrimeScript RT Master Mix kit (Takara Biotechnology, Co., Ltd.). Subsequently, standardized quantitative PCR was performed using Premix Ex Taq™ (Takara Bio, Inc.) in 384-well plates and an Applied Biosystems Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the following thermocycling conditions were used: Initial denaturation at 95˚C for 10 min, 95˚C for 20 sec and 60˚C for 45 sec (for 40 cycles). The 16 genes examined comprised of five proliferation-related genes [Ki-67, aurora kinase A (AURKA), baculoviral IAP repeat containing 5 (BIRC5), cyclin B1 (CCNB1) and MYB proto-oncogene like 2 (MYBL2)], two metastasis-related genes [MMP11 and cathepsin V (CTSV)], two HER2-related genes [growth factor receptor bound protein 7 (GRB7) and HER2], four hormone-related genes [ER, PR, BCL2 and signal peptide CUB domain and EGF like domain containing 2 (SCUBE2)] and three independent genes [glutathione S-transferase mu 1 (GSTM1), BAG cochaperone 1 (BAG1) and CD68], which were normalized according to five reference genes (ACTB, GAPDH, RPLP0, GUSB and TFRC). Therefore, 16 cancer-related genes in 21 genes can be used to predict the outcome of patients. The expression of the genes was confirmed in triplicate, and the relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (22), and the RS was calculated based on the Oncotype DX formula (10). According to the RS results, patients were categorized into low-risk (RS <18), intermediate-risk (RS ≥18-30) and high-risk (RS ≥30) groups (23). For further analysis, the individual gene expression of the 16-cancer genes was measured, and the distribution of the 16-cancer gene expression in PMBC and MMBC cases was analyzed.

Statistical analysis. The clinicopathological characteristics were presented as patient number and percentage and the other data was expressed as the mean ± standard deviation and range. The χ^2 test or Fisher's exact test were used to evaluate associations between PMBC and MMBC, while the Kruskal-Wallis test was used to compare quantitative characteristics. Logistic regression was used in multivariate analyses to identify risk factors impacting lymph node metastasis. The Kaplan-Meier estimation (log-rank test) was used to assess DFS and OS rate, and the Cox proportional hazard model was used to analyze the prognostic factors of patients with MBC. The Mann-Whitney test was used to assess the distribution of the 21-gene RS in the different subgroups, and to compare the expression levels of the 16 cancer genes between subgroups. $P<0.05$ was considered to indicate a statistically significant difference. SPSS version 22.0 software (IBM Corp.) was used for all the statistical analyses.

Results

Patients and baseline clinicopathological features. In total, 49 cases diagnosed as MBC (38 PMBCs and 11 MMBCs) were included in this analysis and the pathological changes of various typical MBCs are shown in Fig. 1. The median age at diagnosis was 52.3 ± 12.8 years (range, 33-87 years), and 44.9% of these patients were postmenopausal. The median tumor size was 3.2 ± 1.8 cm (range, 1.0-8.5 cm) at diagnosis, and 29.2% of cases had axillary lymph node involvement. According to IHC and FISH results, 45 (91.8%) and 38 (77.6%) patients with MBC were ER and PR positive, respectively. In 5 (10.2%) of the patients with MBC, HER2 positivity was detected, while 34.7% of all patients had ≥20% Ki-67 expression. For the molecular subtype, 49.0% (n=24) were classified as luminal A, 42.8% (n=21) as luminal B, 4.1% (n=2) as HER2-rich and 4.1% (n=2) as triple negative. The detailed clinicopathological characteristics of the patients are shown in Table II.

The mean age at diagnosis in patients with PMBC and MMBC was 51.5 ± 13.4 years (range, 33-87 years) and 54.9 ± 10.8 years (range, 33-78 years), respectively (P=0.25), and the mean tumor size in PMBCs and MMBCs was 3.19 ± 1.8 cm (range, 1.2-8.5 cm) and 3.17 ± 1.6 cm (range, 1.0-5.0 cm), respectively (P=0.914). The data showed no significant differences between PMBCs and MMBCs with respect to TNM stage (P=0.261), molecular subtype (P=0.17), status of ER (P=0.562), status of PR (P=0.398) and Ki-67 expression (P=0.395). However, a significantly higher incidence rate of axillary lymph node involvement was observed in MMBCs comparison with that in PMBCs (72.7 vs. 16.2%, respectively; P=0.001). The clinicopathological characteristics of the PMBCs and MMBCs are detailed in Table III. Similarly, the results of multivariate analysis demonstrated that the only high-risk factor of lymph node metastasis in patients with MBC was the pathological subtype (P=0.018; Table IV). Furthermore, the status of HER2 had a marginal P-value (P=0.068) in the two groups, and a higher incidence rate was observed in MMBCs compared with that in PMBCs (27.3 vs. 5.3%).

Treatment and prognosis in patients with MBC. A total of 98.0% of the patients with MBC in the present study underwent radical mastectomies (1 patient refused surgery), and the

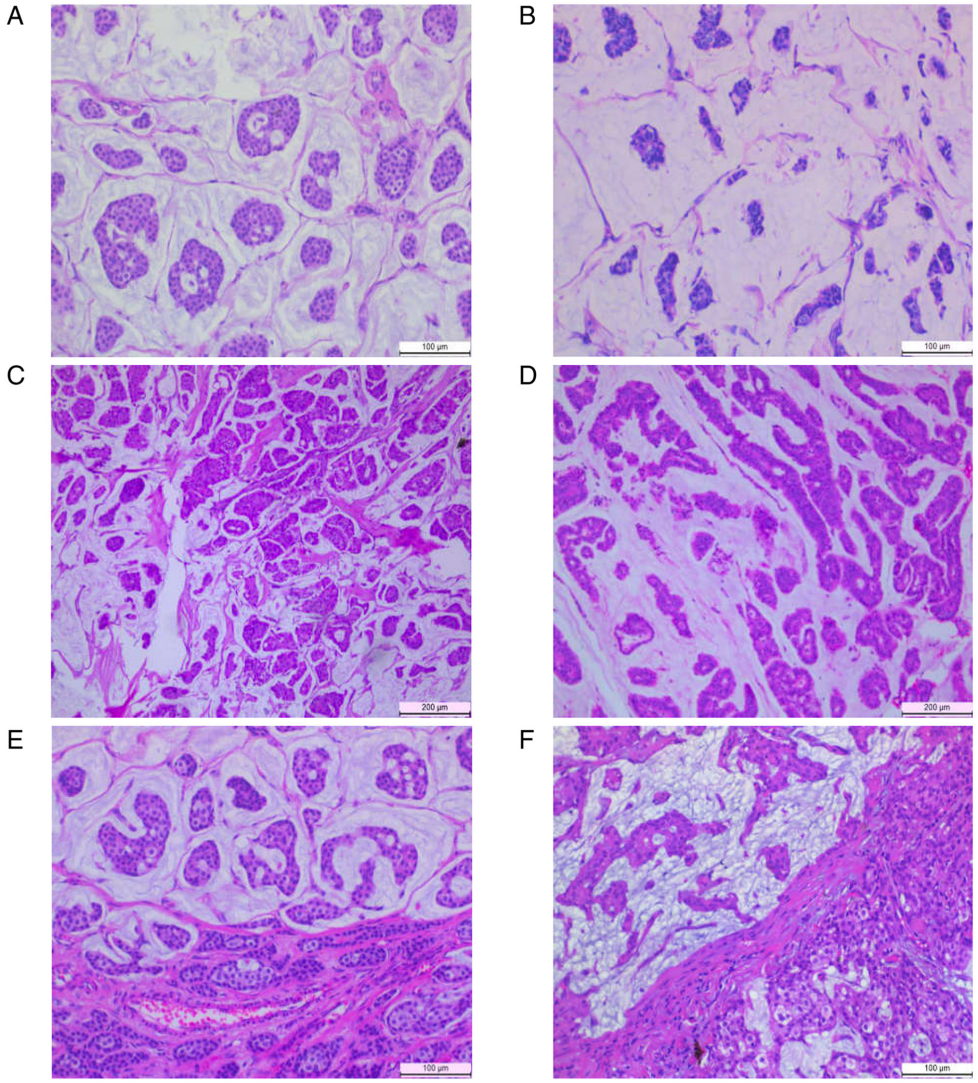

Figure 1. Histological features of different subtypes of breast mucinous carcinoma. (A and B) Type A (Paucicellular) variant. (C and D) Type B (hypercellular) variant. (E and F) mixed mucinous carcinoma variant.

first-line treatment selections following surgery in the MBC cases with different subtypes are presented in Fig. 2A. Overall, 8.2% (n=4), 36.7% (n=18) and 51% (n=25) of enrolled patients received CT, endocrine therapy (ET) and CT followed by ET as first-line treatment according to the molecular subtypes, respectively. The detailed adjuvant treatment of the MBC cases with various molecular subtypes are shown in Table I. Of all the patients with HER2 expression amplification, only 1 patient (20%) received trastuzumab therapy. In the PMBC and MMBC cases, the proportion of those receiving CT was 55.3 and 72.7%, respectively, and there was no statistical significance (P=0.102; data not shown).

The mean follow-up time for patients with MBC was 65.6 months (range, 2-125 months), and 2 patients were lost during this time. As shown in Fig. 2B and C, the 5-year DFS and 5-year OS rates for MBC was 97 and 100%, respectively, and this result was not statistically significant between PMBCs and MMBCs (log-rank test; P=0.457).

During the study period, distant metastases were found in 5 patients with high TNM stage (3 cases with stage III and 2 cases with stage II), and 2 of these patients died from lung metastases (both HER2 expression positive). In addition, 1 patient with PMBC with no recurrence died of a cardiovascular accident. The causes of treatment failure in MBCs cases are presented in Table **V. In addition, Cox multi**-variate analysis did not identify any statistically significant factors associated with the prognosis of patients with MBC (Table VI).

Comparison of Oncotype DX 21-gene RS and individual gene expression between the PMBC and MMBC groups. In the present study, 29 of the 42 enrolled ER-positive and HER2-negative MBC cases underwent Oncotype DX 21-gene testing (the sample quality of 13 cases did not meet the test) and the results were evaluable, which included 21 PMBCs and 8 MMBCs. According to the criteria of 21-gene test RS stratification, 51.7% patients (15/29) were in the low-risk group (RS <18) with a mean RS of 10.5 ± 5.6, 44.8% patients (13/29) were in the intermediate-risk group (RS ≥18-30) with a mean RS of 22.3±5.2, and 3.5% patients (1/29) were in the high-risk group (RS ≥30) (RS, 35.7). The proportions of low-, intermediate- and high-risk RS were 42.9, 52.3 and 4.8%,

Table II. Clinicopathological features of patients with mucinous breast carcinoma (n=49).

Parameters	Number (%)
Age, years	
≤50	26 (53.1)
≥50	23 (46.9)
Menstruation	
Premenopausal	27 (55.1)
Postmenopausal	22 (44.9)
Tumor size, pT[a]	
T1	18 (37.5)
T2	18 (37.5)
T3	12 (25.0)
Nodal status, pN[a]	
N0	34 (70.8)
N1	7 (14.6)
N2	6 (12.5)
N3	1 (2.1)
TNM stage[a]	
I	13 (27.1)
II	26 (54.2)
III	9 (18.7)
Subtype	
PMBC	38 (77.6)
MMBC	11 (22.4)
ER status	
Positive	45 (91.8)
Negative	4 (8.2)
PR status	
Positive	38 (77.6)
Negative	11 (22.4)
HER2 status	
Positive	5 (10.2)
Negative	44 (89.8)
Ki67, %	
<20	32 (65.3)
≥20	17 (34.7)
Molecular subtype[b]	
Luminal A	24 (49.0)
Luminal B[c]	21 (42.8)
HER2	2 (4.1)
Triple negative	2 (4.1)

[a]There was one patient did not receive surgery. [b]The Molecular subtype was defined based on the 2013 St. Gallen consensus. [c]This includes 11 patients who are ER-positive and HER2-negative and 3 patients who are ER-positive and HER2-positive. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PMBC, pure mucinous breast carcinoma; MMBC, mix mucinous breast carcinoma.

respectively, among PMBCs, and 50.0, 50.0 and 0% in the MMBCs (P=0.91; Fig. 3A). The mean 21-gene RS in the PMBC and MMBC groups was 18.0 and 13.0, respectively (P=0.151; Fig. 3B). Notably, based on the traditional RS treatment recommendation, 37.9% of patients with MBC in the present study could avoid CT (Fig. S1).

The individual gene expression of the 16 cancer genes from the 21-gene test between the PMBC and MMBC groups was analyzed. The histograms of the distribution of cancer gene expression in the different histological-type subgroups are presented in Fig. 3C. In general, the expression levels of the genes from the proliferation and HER2 groups did not differ significantly between the PMBC and MMBC cases. In the metastasis group, the expression level of CTSV (P=0.005) was significantly higher in the PMBC group compared with that in the MMBC group. In the ER group, the expression level of PR (P=0.018) was significantly higher in the PMBC group compared with that in the MMBC group, and the expression level of ESR1 had a marginal P-value (P=0.053). Furthermore, in the independent group, the expression level of CD68 was higher in the PMBC group (P=0.003), while the expression levels of GSTM1 and BAG1 did not differ significantly between groups. The detailed expression of the 16 cancer genes between the PMBC and MMBC groups are shown in Table VII.

Discussion

MBC is a rare histological type of primary breast cancer and a previous epidemiological survey reported that the incidence rate of MBC in Caucasians was lower compared with that in Africans (24). Prior studies indicated that the majority of MBC cases were ER-positive, HER2-negative tumors without node metastasis, which suggested that the treatment of MBC should be different from IDC (14). Therefore, it is necessary to divide patients into different subgroups according to the recurrence risk and it can be used to choose more reasonable adjuvant therapy. The 21-gene RS has been proved to assist clinicians with therapeutic decisions; however, data on the RS of MBC remains limited and to the best of our knowledge, this topic has not been addressed in large studies. The present study assessed the clinicopathological features, treatment and prognosis of patients with MBC. More importantly, it evaluated the distribution pattern and clinical value of the 21-gene RS in patients with MBC. To the best of our knowledge, the current study represents the first study focused on comparing the 21-gene RS and individual gene expression for patients with PMBC and those with MMBC.

In the current study, from the 3,081 patients with invasive breast cancer, 49 (1.59%) had MBC and the incidence rate was similar to that of other studies (1,2,25). The present results demonstrated that postmenopausal women accounted for 44.9% of all MBCs, and 91.8% (45/49) and 77.6% (38/49) MBC cases were ER- and PR-positive, respectively, which were consistent with previous findings (3,4). In addition, 29.2% of MBCs had axillary lymph node metastases, which was higher than the incidence rate of axillary metastases, ranging from 3-26%, reported in the literature (1,2,4,5). This may be because 22.4% of the cases (11/49) in the current study were MMBCs. Next, the present study compared the clinicopathological characteristics of patients with PMBC and those with MMBC. There were no significant differences between

Table III. Comparison of clinicopathological characteristics in patients with PMBC and MMBC.

Characteristic	PMBC	MMBC	P-value
Mean age ± SD (range), years	51.5±13.4 (33-87)	54.9±10.8 (39-78)	0.25
Mean tumor size ± SD (range), cm	3.19±1.8 (1.2-8.5)	3.17±1.6 (1.0-5.0)	0.914
Nodal status, pN[a]			0.001
N0	31	3	
N1-3	6	8	
TNM stage[a]			0.261
I	11	2	
II	21	5	
III	5	4	
ER status			0.562
Positive	34	11	
Negative	4	0	
PR status			0.398
Positive	31	7	
Negative	7	4	
HER2 status			0.068
Positive	2	3	
Negative	36	8	
Ki67, %			0.395
<20	26	6	
≥20	12	5	
Molecular subtype[b]			0.17
Luminal A	21	4	
Luminal B	14	7	
HER2	2	0	
Triple negative	2	0	

[a]There was one patient did not receive surgery. [b]The Molecular subtype was defined based on the 2013 St. Gallen consensus. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PMBC, pure mucinous breast carcinoma; MMBC, mix mucinous breast carcinoma.

PMBCs and MMBCs with respect to age, tumor size, TNM stage, ER status, PR status and Ki-67 expression. However, patients with MMBC showed a significantly higher incidence rate of axillary nodal metastases compared with those with PMBC (72.7 vs. 16.2%), which was consistent with previous studies (8,26). Notably, a higher incidence rate of HER2 positivity was observed in MMBCs in comparison with PMBCs (27.3 vs. 5.3%), and this phenomenon has been confirmed by other study (27).

The present study also assessed the treatment and prognosis in PMBC and MMBC cases. According to the clinical stage and molecular subtype, 55.3% of PMBCs and 72.7% of PMBCs received CT, while the proportion of PMBC and MMBC cases receiving ET was 84.2 and 100.0%, respectively. With a mean follow-up of 65.6 months (range, 2-125 months), it was demonstrated that patients with MBC had excellent 5-year DFS (97.0%) and OS (100.0%) rates, which was similar to findings of other studies (3,28,29). However, the difference in the 5-year DFS and OS rates between PMBCs and MMBCs were statistically insignificant, which was not consistent with previous studies (8,17). This phenomenon could be explained by the relatively short follow-up time and small number of patients with metastasis and those that died.

In the present study, Oncotype DX 21-gene testing was performed in 29 ER-positive/HER2-negative patients with MBC, including 21 PMBCs and 8 MMBCs. The results indicated that 51.7% of MBC cases were in the low-risk group, with a mean RS of 10.5 ± 5.6, although 4 patients (26.7%) had lymph node metastases. The intermediate-risk group included 13 patients with MBC, which had a mean RS of 22.3 ± 5.2, and 5 patients (28.5%) in this group had lymph node metastases. In addition, only 1 node-negative cases was classified into the high risk group, with a mean RS of 35.7. These results showed a lower proportion of patients with low-risk and a higher proportion of patients with intermediate-risk compared with that in the study by Turashvili *et al* (29), which may be due to the fact that the patients included in the current study have more high-risk clinical factors. Based on the traditional RS treatment recommendation, 37.9% of patients with MBC in the

Table IV. Logistic regression analysis of factors predicting lymph node metastasis.

Parameters	B	S.E	Wald	P-value	95% CI	
					Lower	Upper
Age (<50 vs. >50 years)	-1.274	0.928	1.885	0.170	0.045	1.724
Tumor size (<2 vs. >2 cm)	0.791	0.858	0.849	0.375	0.410	11.853
ER (positive vs. negative)	19.138	17425.283	0.000	0.999	N/A	N/A
PR (positive vs. negative)	0.056	1.116	0.030	0.960	0.119	9.419
HER2 (positive vs. negative)	20.274	17425.283	0.000	0.999	N/A	N/A
Ki67 (<20 vs. >20%)	0.809	0.938	0.743	0.389	0.357	14.116
Subgroup (PMBC vs. MMBC)	2.629	1.116	2.527	0.018	1.556	123.560
Constant	-21.074	17425.283	0.000	0.999		

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; B, regression coefficient; S.E, standard error; Wald, χ^2 value; N/A, not available; CI, confidence intervals.

Table V. Disease recurrence and survival profile of the enrolled patients.

Recurrence/metastasis sites	Pathological subtype	Molecular subtype	Stage	TTR[a], month	Outcome
Chest wall and lung	MMBC	B/HER2	T3N2M0	50	Death
Lung	PMBC	B	T2N0M0	76	Survival
Bone and lung	MMBC	B/HER2	T3N1M0	79	Death
Bone	PMBC	A	T3N2M0	72	Survival
Bone	PMBC	A	T2N0M0	58	Survival

[a]Time since surgery until diagnosis of recurrence. TTR, time to relapse; A, luminal A subtype, B, luminal B subtype, HER2, human epidermal growth factor receptor 2 subtype; PMBC, pure mucinous breast carcinoma; MMBC, mix mucinous breast carcinoma.

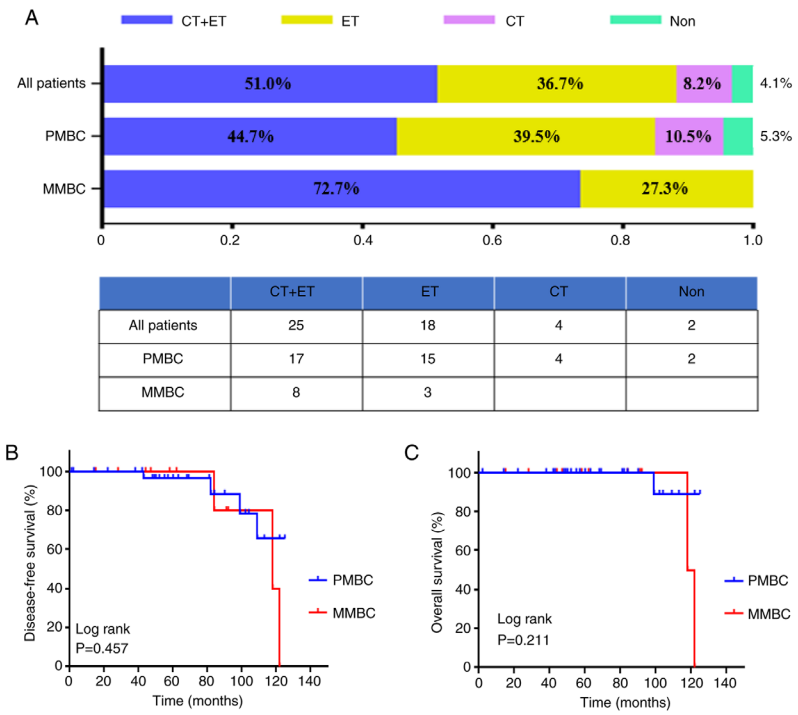

Figure 2. Treatment and prognosis of MBC. (A) Distribution of systemic treatment following surgery. The probability of (B) disease-free survival and (C) overall survival in relation to the subtypes, PMBC and MMBC. PMBC, pure mucinous breast carcinoma; MMBC, mixed mucinous breast carcinoma; CT, chemotherapy; ET, endocrine therapy; Non, no endocrine therapy and chemotherapy.

Table VI. Prognostic significance of the clinicopathological factors on DFS and OS in patients with MBC.

	DFS			OS		
		95% CI			95% CI	
Parameters	P-value	Lower	Upper	P-value	Lower	Upper
Age (<50 vs. >50 years)	0.239	0.220	2.548	0.975	0.280	32.050
Tumor size (<2 vs. >2 cm)	0.939	N/A	N/A	0.945	0.410	11.853
Nodes (positive vs. negative)	0.986	0.940	10.256	0.953	N/A	N/A
Subgroup (PMBC vs. MMBC)	0.952	N/A	N/A	0.999	N/A	N/A
ER (positive vs. negative)	0.980	N/A	N/A	0.990	N/A	N/A
PR (positive vs. negative)	0.960	N/A	N/A	0.969	0.119	9.419
HER2 (positive vs. negative)	0.944	N/A	N/A	0.966	N/A	N/A
Ki67 (<20 vs. >20%)	0.498	0.830	3.361	0.830	0.051	10.836

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; DFS, disease-free survival; OS, overall survival; N/A, not available; CI, confidence intervals; MBC, mucinous breast carcinoma.

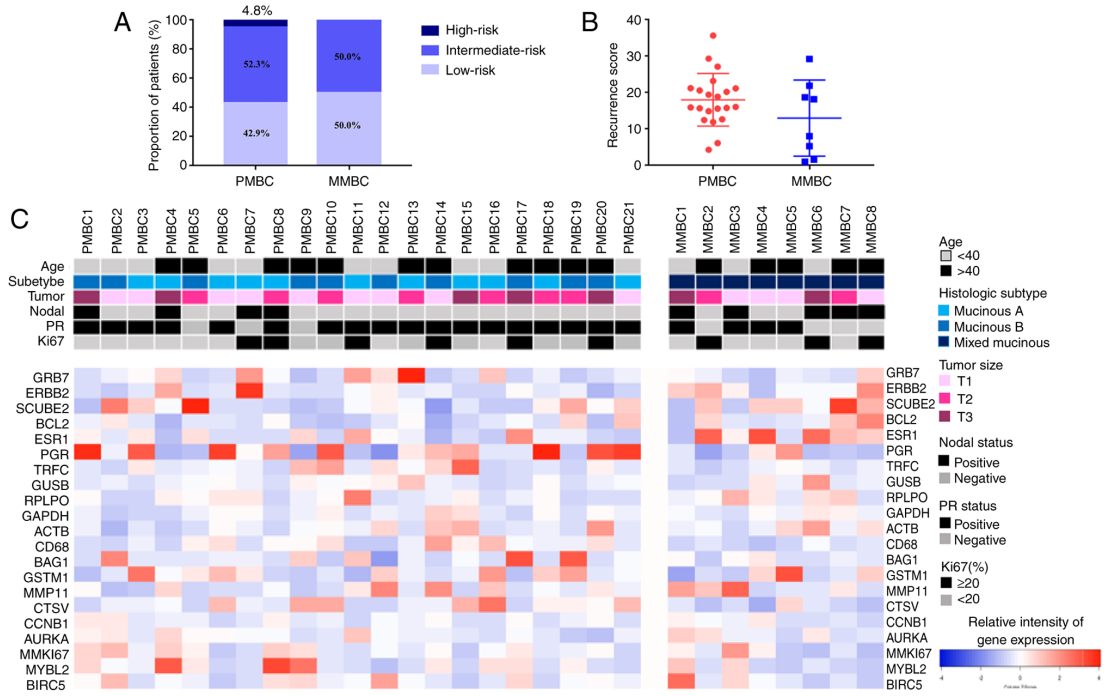

Figure 3. Expression levels of the 21-genes in mucinous breast carcinomas. (A) Proportion of the 21-gene risk stratification in PMBC and MMBC cases (P=0.91). (B) Distribution of the 21-gene RS in PMBCs and MMBCs (P=0.151). (C) Individual expression levels of the 16 cancer genes from the 21-gene RS identified in PMBCs (n=21; left) and in the MMBC (n=8; right). Histopathological characteristics are depicted in the phenotype bars (top) and the relative intensity of gene expression is shown in the heat map. PMBC, pure mucinous breast carcinoma; MMBC, mixed mucinous breast carcinoma; RS, recurrence score; PR, progesterone receptor.

present study could avoid CT, and 27.6% of them could choose CT or not. Notably, the NSABP B-20 study reported that only patients which had a RS ≥31 benefited the most from adjuvant CT (30), and the TAILORx study only recommended that patients with a RS ≥26 receive adjuvant CT (31). Furthermore, the Southwest Oncology Group-8814 and Eastern Cooperative Oncology Group E2197 studies extended the application of the 21-gene RS assay to the lymph node positive population, as well as advocated RS use in patients with 1-3 positive lymph nodes and considered omitting adjuvant CT in those with a RS <18 (12,13). However, this requires further research for confirmation. In addition, previous research has analyzed the association between RS and the prognosis of MBC and found that it is no significant differences in DFS and OS rates among MBC patients in different RS risk groups (15). However, it is difficult to analyze the association between RS and prognosis in the study as only 2 patients had metastasis (PMBC14, RS, 18.87 and PMBC2, RS, 15.63).

Table VII. Comparison of individual gene expression levels of the 16 cancer genes from the 21-gene RS in patients with PMBC and MMBC.

A. Proliferation group

Gene name	AEI in PMBC ± SD (range)	AEI in MMBC ± SD (range)	P-value
CCNB1	0.93±1.17 (0.5-5.58)	1.26±0.41 (0.41-1.53)	0.793
AURKA	1.23±0.76 (0.44-3.9)	1.0±0.59 (0.41-1.98)	0.649
MKI67	1.56±1.75 (0.31-8.45)	1.15±1.29 (0.21-4.16)	0.324
MYBL2	2.27±4.45 (0.3-21.27)	0.94±0.93 (0.21-2.67)	0.168
BIRC5	1.48±1.49 (0.17-7.06)	1.22±1.14 (0.24-3.46)	0.401

B. Invasion group

Gene name	AEI in PMBC ± SD (range)	AEI in MMBC ± SD (range)	P-value
MMP11	1.2±0.98 (0.28-4.07)	1.82±1.66 (0.47-5.38)	0.324
CTSV	1.59±1.37 (0.42-6.62)	0.61±0.48 (0.16-1.6)	0.005

C. ER group

Gene name	AEI in PMBC ± SD (range)	AEI in MMBC ± SD (range)	P-value
SCUBE2	1.53±1.66 (0.14-6.24)	1.92±1.84 (0.43-6.06)	0.401
BCL2	1.29±1.26 (0.31-5)	1.37±1.00 (0.5-2.94)	0.457
ESR1	1.22±1.07 (0.31-4.98)	2±1.12 (0.48-3.65)	0.053
PGR	2.83±3.19 (0.05-12.9)	0.7±0.86 (0.04-2.53)	0.018

D. HER2 group

Gene name	AEI in PMBC ± SD (range)	AEI in MMBC ± SD (range)	P-value
GRB7	2.04±3.12 (0.35-14.5)	0.91±0.51 (0.43-1.86)	0.457
ERBB2	1.26±1.62 (0.32-7.96)	1.57±0.90 (0.4-2.84)	0.103

E. Independent group

Gene name	AEI in PMBC ± SD (range)	AEI in MMBC ± SD (range)	P-value
CD68	1.38±10.8 (0.32-4.66)	0.63±0.22 (0.42-1.04)	0.003
BAG1	1.6±1.83 (0.36-6.7)	0.96±0.44 (0.43-1.74)	0.684
GSTM1	1.62±1.66 (0.14-5.9)	1.13±1.17 (0.09-3.63)	0.457

AEI, average expression intensity; PMBC, pure mucinous breast carcinoma; MMBC, mix mucinous breast carcinoma.

Next, the current study performed a comparison of the 21-gene RS between PMBCs and MMBCs and the data revealed there was no statistically significant differences between the two groups. This result suggests that PMBCs and MMBCs may have similar 21-gene RS with the same molecular subtypes ($ER^+/HER2^-$), but larger sample studies are required to confirm this conclusion. Analysis of the individual cancer gene expression differences from the 21-gene RS between PMBCs and MMBCs was performed, and three of these genes were differently expressed in PMBC compared with MMBC. As a key element in tumor growth and metastasis, a high expression level of CTSV was previously shown to be associated with poor prognosis in breast cancer (32). In the current study, the expression of CTSV was significantly higher in PMBCs compared with that in MMBCs, which suggested that the cell invasive ability of the former may be higher compared with that of the latter. However, this phenomenon is not consistent with the fact that the lymph node metastasis rate of patients with MMBC was higher compared with that in patients with PMBC and further studies are required

to verify the association between CTSV and MBC. PR is the main downstream signal molecule in the ER signaling pathway (33), and PR status was defined as a predictor for RS according to previous analyses in the Plan B and NASBP B20 studies (30,34). The present data revealed that the expression level of PR in the PMBC group was significantly higher compared with that in the MMBC group, which suggested that PMBC had a more favorable response to ET. CD68 is a marker of macrophages and its expression can indicate the infiltration of tumor lymphocytes (35). A previous study confirmed that a high level of CD68 protein expression was associated with poor prognosis in patients with breast cancer (36). In the current study, the expression level of CD68 was higher in the PMBC group compared with the MMBC group, which indicated that the immune status was different between the two groups, which warrants further investigation.

The current study has some limitations. First, the number of MBC cases was limited due to its relatively low incidence. Second, the study was single-centered and retrospective, which could cause selection bias. Finally, the follow-up time was relatively short and ongoing, and a longer follow-up would be of benefit for further conclusions for MBC.

In conclusion, the main purpose of the present study was to evaluate the distribution pattern and clinical value of 21-gene RS in patients with MBC. The clinicopathological data and prognosis of 38 patients with PTMC and 11 patients with MMBC were analyzed and a total of 29 ER-positive and HER2-negative patients with MBC underwent the Oncotype DX 21-gene test. The results showed patients with MBC had favorable prognosis, and patients with PMBC and MMBC had low- and intermediate-risk RS, which suggested that a considerable proportion of patients may be able to avoid CT; however, further research and clinical trials should be conducted to confirm the observations. There were no statistically significant differences between PMBCs and MMBCs in the 21-gene RS, but the high expression level of PR-related genes in PMBCs indicated that they may have an improved response to ET compared with MMBCs. In addition, CTSV and CD68 expression showed a significant difference between the PMBC and MMBC groups, which may indicate that they have different tumor characteristics and further studies are required to verify the association of these gene expression patterns on MBC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RC, YW, JW and XC conceived the study. RC, YW, TL, JL, NT and GF collected and interpreted the data. JW and XC confirm the authenticity of the raw data. RC, YW, NT and JW performed the data analysis. RC and YW wrote the manuscript. XC, TL and JL reviewed and edited the manuscript. RC, TL and JL acquired the funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were performed in accordance with the ethical standards of the Ethical Committees of Affiliated Hospital of Zunyi Medical University and the Declaration of Helsinki of 1964. The present study was reviewed and approved by the Ethical Committee of Affiliated Hospital of Zunyi Medical University. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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