Quantification of Ki67 Change as a Valid Prognostic Indicator of Luminal B Type Breast Cancer After Neoadjuvant Therapy

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Introduction: Ki67 value and its variation before and after neoadjuvant chemotherapy are commonly tested in relation to breast cancer patient prognosis. This study aims to quantify the extent of changes in Ki67 proliferation pre- and post-neoadjuvant chemotherapy, confirm an optimal cut-off point, and evaluate its potential value for predicting survival outcomes in patients with different molecular subtypes of breast cancer.

Methods: This retrospective real-world study recruited 828 patients at the Department of Breast Surgery of the First Affiliated Hospital of China Medical University and the Cancer Hospital of China Medical University from Jan 2014 to Nov 2020. Patient demographic features and disease pathology characteristics were recorded, and biomarkers were verified through immunohistochemistry. Various statistical methods were used to validate the relationships between different characteristics and survival outcomes irrespective of disease-free and overall survival.

Results: Among 828 patients, statistically significant effects between pathological complete response and survival outcome were found in both HER2-enriched and triple-negative breast cancer (p < 0.05) but not in Luminal breast cancer (p > 0.05). Evident decrease of Ki67 was confirmed after neoadjuvant chemotherapy. To quantify the extent of Ki67 changes between pre- and post-NAC timepoints, we adopted a computational equation termed ΔKi67% for research. We found the optimal cut-off value to be “ΔKi67% = −63%” via the operating characteristic curve, defining ΔKi67% ≤ −63% as positive status and ΔKi67% > −63% as negative status. Patients with positive ΔKi67% status were 37.1% of the entire cohort. Additionally, 4.7, 39.9, 34.5 and 39.6% of patients with Luminal A, Luminal B, HER2-enriched and triple negative breast cancer were...
INTRODUCTION

Breast cancer is the highest cause of cancer-related morbidity among women worldwide [1]. Established biomarkers, including hormone receptors (HR), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor-2 (HER-2), and Ki67 labeling index classify breast cancer into four subtypes: HER2-enriched, triple-negative (TN), and Luminal A and B types [2,3]. Neoadjuvant chemotherapy (NAC) is a standard therapeutic strategy for inoperable breast cancer and for some operable patients who seek decreased primary tumor burden and breast conservation [4]. Patient response to NAC also provides guidance for the long-term systemic therapeutic strategy for each individual patient [5]. A achievement of pathological complete response (pCR), disease-free survival (DFS), and overall survival (OS) [6] were used to estimate treatment efficacy. Only 15–20% of patients who receive NAC reach pCR [7–9]. Although pCR plays an important role in prognostic prediction and assists in treatment decisions for TN and HER2-enriched breast cancer, it is less effective in Luminal breast cancer subtypes [10–12]. Luminal breast cancer still lacks indicators to classify patients who will benefit from NAC.

Ki67, a nuclear indicator of cellular proliferation, has been extensively studied and scrutinized for several years. Although some studies criticize Ki67 for its lack of reproducibility [13,14], many demonstrate that proliferation index relates to patient outcomes [2,4,15–17]. These study show Ki67 expression as useful indicator for breast cancer and a useful prognostic factor for patients with Luminal B and node-positive breast cancer, assisting in clinical decision regarding neoadjuvant endocrine therapy [18,19]. Some studies indicate that Ki67 levels pre-NAC can be an independent prognostic predictor for OS and DFS [12,16]. Endocrine therapy can decrease cell proliferation, presenting as changed Ki67 level pre- and post-NAC [20,21]. In the POETIC clinical phase-3 trial, this change in Ki67 levels was able to guide endocrine therapy decisions for women with ER-positive breast cancer [19]. One commonality across studies was that decreased levels of Ki67 post-NAC compared to pre-NAC holds significant prognostic predictive value [12,18,22–25]. However, some researchers contend that post-NAC Ki67 may hold limited prognostic value [26].

Previous researches often define the extent of Ki67 change before and after NAC as simply by subtracting the two values. This definition is simple but insufficient, as illustrated in two scenarios. The first is if pre- and post-NAC Ki67 proliferation are both relatively low, then the extent of the change may not reach the set threshold. In the second, the change may be comparatively large but not large enough to reach the cut-off value. Furthermore, high variation of pre- and post-NAC Ki67 have been classified by several groups, with studies proposing different thresholds of variation based on the attempts.

In this retrospective study, we evaluate the usefulness of Ki67 change before and after NAC for predicting survival outcome across breast cancer molecular subtypes. We further quantify the change in Ki67 by percentage before and after NAC and calculate an optimal threshold to assess its predictive function for long-term survival and its ability to aid in deciding further adjuvant therapy modification across breast cancer subtypes.

METHODS

Patient Selection Criterion

This retrospective study included patients with primary breast cancer who were treated with NAC from January 2014 to November 2020 at the Department of Breast Surgery of the First Affiliated Hospital of China Medical University and the Cancer Hospital of China Medical University.

Patient Inclusion Criteria

All patients received a minimum of one cycle of NAC ahead of surgery. Patients with cancer in situ were excluded, as were patients with invasive breast cancer before NAC could be incorporated in cohort. Patients who received any kind of treatment prior to NAC or who presented with progressive or metastatic breast cancer were excluded. Patients with previous breast cancer, male patients, and those with synchronous invasion, bilateral, or inflammatory breast cancer were also excluded. 68 cases with incomplete or deficient IHC analysis were also excluded. In total, 828 patients met the above restriction standards and were included.
Classifications of Patients
This retrospective study received permission from the institutional review board (IRB) of the First Affiliated Hospital and was in accordance with the Helsinki Declaration. All patients involved in the research gave informed consent in written agreements of specimens used for scientific research. The informed consent of retrospective research involvement could be waived based on the retrospective nature of the study. Pre-NAC core needle biopsy pathology and post-surgery regular pathology was extracted and saved in a database. Patient characteristics were collected including gender, age at diagnosis, body mass index (BMI), maximum tumor diameter, tumor grade and stage, axillary lymph node status, histologic type, NAC schedule and cycle number, histology grade, and clinical response to NAC. The local extent of breast cancer was measured via breast ultrasound, mammography, breast MRI, chest CT, bone scan and/or hepatobiliary and splenic ultrasound to verify distant metastasis. The final size of local breast cancer in our database was adopted following priorities: breast MRI > breast ultrasound > mammography. Every patient with suspicious lymph-node metastasis suggested by imaging examinations underwent ultrasound-guided core biopsy of ultrasound-graphically abnormal nodes for axillary node metastasis confirmation before starting NAC. The final histological assessments were all analyzed using hematoxylin and eosin staining and immunohistochemical (IHC). The histological type of specimens from incorporated patients was distinguished between two subgroups: general invasive breast carcinoma of no special type (IBC-NST) and Others. The latter group contained special subtypes such as lobular, mucinous and carcinoma of no special type (IBC-NST) and Others. The latter histological type of specimens from incorporated patients was histological assessments were all analyzed using hematoxylin and eosin staining. IHC staining was performed using Dako Autostainer Plus and EnVision Dual Link detection reagent (DAKO; Carpinteria, CA) with DAB (Dako). Biomarker status, including ER, PR and HER2, were defined by IHC in strict accordance with European Quality Assurance guidelines. ER and PR staining were assessed based on the American Society of Clinical Oncology/College of American Pathologists Guidelines [27,28]. Antibodies used in IHC include as anti-ER (Clone SP1, Dako), anti-PR (Clone PgR636, Dako), and Ki67 (Clone Mib-1, Dako). Hematoxylin II (Dako As Link 48) was used to counterstain specimens automatically. All tests incorporated external positive and negative controls. ER and PR stains were considered positive if immunostaining was seen in more than 1% of immunoreactive cells. HER2 status was ascertained via IHC using the Hercep Test™ kit (code K5204, Dako). HER2 expression was scored as 0, 1+, 2+, and 3 + according to ASCO guidelines. A score of 3+ was regarded as HER2+, with 0/1+ defined as HER2-. For cases scoring HER2 2+, a fluorescent in situ hybridization (FISH) test could be conducted. The measurement of Ki67 index was based on the spot with the highest intensity in a high-power field (400x) and 500–2000 cells were counted [29].

Following the St. Gallen guidelines 2013 [2], high expression of PR was set as ≥20% and low expression of PR was defined as <20%. In accordance with the International Ki67 in Breast Cancer Working Group [30], Ki67 index was classified into two groups: low (<30%), and high (≥30%). To quantify the extent of Ki67 changes between pre- and post-NAC timepoints, we used the following equation: [define post-NAC Ki67 as A, define pre-NAC Ki67 as B, computational formula: ΔKi67%=(A−B)/B × 100%, maintaining sign]. If a patient achieved pCR after NAC, the post-NAC Ki67 index was defined as 0% and ΔKi67% was mathematically ~100%. Representative IHC staining images of Ki67 subgroups are shown in Supplementary Figure S2.

Assessment of Clinical Effectiveness of Chemotherapy
Pathological complete response (pCR) was defined as no residual invasive tumor upon hematoxylin and eosin evaluation of the complete resected breast specimen and all sampled lymph nodes (noninvasive breast residuals) (ypT0/is, ypN0).

Immunohistochemistry for Biomarker Detection
Histopathology is regarded as the gold standard for diagnosis. All breast tumor specimens were acquired from core needle biopsy or surgical resections, and every specimen was affixed into formalin-fixed, paraffin-embedded tissue sections for preservation. IHC staining was performed using Dako Autostainer Plus and EnVision Dual Link detection reagent (DAKO; Carpinteria, CA) with DAB (Dako). Biomarker status, including ER, PR and HER2, were defined by IHC in strict accordance with European Quality Assurance guidelines. ER and PR staining were assessed based on the American Society of Clinical Oncology/College of American Pathologists Guidelines [27,28]. Antibodies used in IHC include as anti-ER (Clone SP1, Dako), anti-PR (Clone PgR636, Dako), and Ki67 (Clone Mib-1, Dako). Hematoxylin II (Dako As Link 48) was used to counterstain specimens automatically. All tests incorporated external positive and negative controls. ER and PR stains were considered positive if immunostaining was seen in more than 1% of immunoreactive cells. HER2 status was ascertained via IHC using the Hercep Test™ kit (code K5204, Dako). HER2 expression was scored as 0, 1+, 2+, and 3 + according to ASCO guidelines. A score of 3+ was regarded as HER2+, with 0/1+ defined as HER2-. For cases scoring HER2 2+, a fluorescent in situ hybridization (FISH) test could be conducted. The measurement of Ki67 index was based on the spot with the highest intensity in a high-power field (400x) and 500–2000 cells were counted [29].

Following the St. Gallen guidelines 2013 [2], high expression of PR was set as ≥20% and low expression of PR was defined as <20%. In accordance with the International Ki67 in Breast Cancer Working Group [30], Ki67 index was classified into two groups: low (<30%), and high (≥30%). To quantify the extent of Ki67 changes between pre- and post-NAC timepoints, we used the following equation: [define post-NAC Ki67 as A, define pre-NAC Ki67 as B, computational formula: ΔKi67%=(A−B)/B × 100%, maintaining sign]. If a patient achieved pCR after NAC, the post-NAC Ki67 index was defined as 0% and ΔKi67% was mathematically ~100%. Representative IHC staining images of Ki67 subgroups are shown in Supplementary Figure S2.

Breast Cancer Subtypes Definitions
We classified breast cancers into four subtypes based on HR status, HER2 status and Ki67 index according to the St. Gallen guidelines as follow [2,31]: Luminal A: (ER and PR positive, HER2 negative, “low” Ki-67, and a “low” recurrence risk based on multi-gene-expression assay results if available), Luminal B (“Luminal B-like (HER2 negative)”: ER positive, HER2 negative, and at least one of the following: “high” Ki-67, “negative or low” PR, or “high” recurrence risk based on multi-gene-expression assay if available. “Luminal B-like (HER2 positive)”: ER positive, HER2 over-expressed or amplified with any Ki-67, and any PR). HER2-enriched
TABLE 1 | Demographic and clinicopathological features of whole cohort (n = 828).

| Parameter | Number (%) |
|-----------|------------|
| Age at diagnosis (year) | |
| <40 | 83 (10.0) |
| ≥40 | 745 (90.0) |
| BMI (kg/m²) | |
| <18.9 (underweight) | 68 (8.2) |
| 18.9–24.9 | 361 (43.6) |
| >24.9 (overweight) | 399 (48.2) |
| Histological type at diagnosis | |
| IBC-NST | 699 (84.4) |
| Others | 129 (15.6) |
| Clinical nodal status at diagnosis | |
| Positive | 676 (81.6) |
| Negative | 152 (18.4) |
| Chemotherapy cycles | |
| ≤2 | 199 (24.2) |
| 3–5 | 424 (51.2) |
| >5 | 205 (24.8) |
| Chemotherapy regimen | |
| Taxane -based | 89 (10.7) |
| Anthracycline-based | 150 (18.1) |
| Taxane + anthracycline | 589 (71.1) |
| Anti-HER2 therapy in patients with HER2-positive (n = 261) | |
| Yes | 49 (18.8) |
| No | 212 (81.2) |
| Clinical tumor stage at diagnosis | |
| T1 | 80 (9.7) |
| T2 | 556 (67.1) |
| T3/T4 | 192 (23.2) |
| Post-NAC tumor size | |
| <2 cm | 434 (52.4) |
| 2–5 cm | 355 (42.9) |
| >5 cm | 39 (4.7) |
| Response to NAC | |
| PR/CR | 494 (59.7) |
| SD/PD | 334 (40.3) |
| Achieved pCR | |
| Yes | 138 (16.7) |
| No | 690 (83.3) |
| ER status* | |
| Positive | 526 (63.5) |
| Negative | 302 (36.5) |
| PR positivity score* | |
| <20% | 520 (62.8) |
| ≥20% | 308 (37.2) |
| HER2 | |
| Positive | 261 (31.5) |
| Negative | 447 (54.0) |
| Unknown | 120 (14.5) |
| Pre-NAC Ki67 | |
| <30% | 253 (30.6) |
| ≥30% | 575 (69.4) |
| Post-NAC Ki67 | |
| <30% | 491 (59.3) |
| ≥30% | 337 (40.7) |
| Molecular subtypes** | |
| Luminal A | 43 (5.2) |
| Luminal B | 489 (59.1) |
| HER2-enriched | 148 (17.9) |
| TNBC | 148 (17.9) |

*Positivity score<1% including negative status.
**Positivity score=20% including negative status.

Luminal A: (ER and PR positive, HER2 negative, "low" Ki-67, and a "low" recurrence risk based on multi-gene-expression assay results if available), Luminal B (Luminal B-like (HER2 negative), ER positive, HER2 negative, and at least one of the following: "high" Ki-67, "negative or low" PR, or "high" recurrence risk based on multi-gene-expression assay if available), Luminal B-like (HER2 positive), ER positive, HER2 over-expressed or amplified with any Ki-67, and any PR; HER2-enriched (HER2 over-expressed or amplified, HR absent) and TN (Negative HR and HER2).

BMI, body mass index; NAC, neoadjuvant chemotherapy; IBC-NST, invasive breast carcinoma of no special Type; pCR, pathological complete response; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer.

Statistical Data Analysis

Multiple demographic features were analyzed using the Chi-square test. The survival-related indicators studied were DFS and OS. DFS was calculated from the date of initiation of the first regimen to the date of first event (locoregional relapse, distant relapse, or death) and OS was calculated from the date of surgery to the date of death or last follow-up. The Kaplan–Meier method was used to define the difference ratio, and survival curves were compared using the log-rank test [32]. Significance was assigned as p value < 0.05. Receiver operating characteristic curve (ROC) and area under the curve (AUC) were performed to calculate the optimal cut-off value determined by the Youden index with maximum sensitivity and specificity. Cox proportional hazards regression models were used to estimate relapse and survival risk between subgroups. The multivariate Cox proportional hazards model was implemented for Hazard Ratio (HR) and 95% confidence intervals (CI) to identify independent prognostic factors. Net reclassification improvement (NRI) was used to verify classification accuracy. All statistical data analysis was performed using SPSS 26.0 (SPSS Inc., Chicago, IL, United States) and R programming language (version 3.5.3; https://www.r-project.org/).

RESULTS

Basic Demographic Features and Baseline Characteristics

Patients with primary breast cancer who were treated with NAC were selected based on strict standards. 942 patients were initially included in the cohort, but 114 patients were eliminated for various reasons. A total of 828 female patients with primary breast cancer who received NAC were ultimately included in this retrospective study (Figure 1).

Basic demographic and pathologic features are shown in Table 1. The median age of entire cohort was 51 ± 9.65 years old (range: 23–76 years), of which 10.0% of patients were under 40 years old at diagnosis. Body mass index was used to distinguish subjects: overweight patients with index greater than or equal to 24.9 accounted for 48.2%, underweight patients with index under 18.9 accounted for 8.2 and 43.6% of patients had a healthy BMI between 18.9–24.9. Breast cancer pathological subtype was...
TABLE 2 | The univariate relationship between above features with pCR (n = 828)

| Parameter                                      | Pathological response to NAC | p Value |
|------------------------------------------------|------------------------------|---------|
| Age at prognosis (years)                       | pCR | Non-pCR |        |
| <40                                            | 18  | 65      | 0.196  |
| ≥40                                            | 120 | 625     |        |
| BMI (kg/m²)                                    |     |         | 0.539  |
| <18.9                                         | 11  | 57      |        |
| 18.9–24.9                                     | 66  | 295     |        |
| >24.9                                         | 61  | 338     |        |
| Histological type                             |     |         | <0.001 |
| IBC-NST                                        | 95  | 604     |        |
| Others                                         | 43  | 86      |        |
| Chemotherapy cycles                            |     |         | 0.432  |
| ≤2                                             | 30  | 169     |        |
| 3–5                                           | 68  | 356     |        |
| >5                                            | 40  | 165     |        |
| Chemotherapy regimen                           |     |         | 0.229  |
| Taxane-based                                   | 13  | 76      |        |
| Anthracycline-based                            | 32  | 118     |        |
| Taxane + anthracycline                         | 93  | 496     |        |
| Anti-HER2 therapy in patients with HER2-positive (n = 261) | | | 0.293 |
| Yes                                            | 12  | 37      |        |
| No                                             | 38  | 174     |        |
| Clinical tumor stage at diagnosis              |     |         | 0.267  |
| T1                                             | 14  | 66      |        |
| T2                                             | 85  | 471     |        |
| T3/T4                                         | 39  | 153     |        |
| Post-NAC tumor size                            |     |         | <0.001 |
| <2 cm                                          | 104 | 330     |        |
| 2–5 cm                                         | 30  | 325     |        |
| >5 cm                                          | 4   | 35      |        |
| Clinical nodal status                          |     |         | <0.001 |
| Positive                                       | 99  | 577     |        |
| Negative                                       | 39  | 113     |        |
| ER status<sup>a</sup>                          |     |         | 0.014  |
| Positive                                       | 75  | 451     |        |
| Negative                                       | 63  | 239     |        |
| PR positivity score<sup>b</sup>                |     |         | 0.028  |
| <20%                                           | 105 | 495     |        |
| ≥20%                                          | 33  | 248     |        |
| HER2                                           |     |         | 0.259  |
| Positive                                       | 50  | 211     |        |
| Negative                                       | 73  | 374     |        |
| Pre-NAC Ki67                                   |     |         | 0.147  |
| <30%                                           | 35  | 218     |        |
| ≥30%                                          | 103 | 472     |        |
| Post-NAC Ki67                                  |     |         | <0.001 |
| <30%                                           | 119 | 372     |        |
| ≥30%                                          | 19  | 318     |        |
| Molecular subtypes<sup>c</sup>                 |     |         | 0.025  |
| Luminal A                                      | 3   | 40      |        |
| Luminal B                                      | 72  | 417     |        |
| HER2-enriched                                   | 29  | 119     |        |
| TNBC                                           | 34  | 114     |        |

<sup>a</sup>Positivity score<1% including negative status.

<sup>b</sup>Positivity score<20% including negative status.

<sup>c</sup>Luminal A: (ER and PR positive, HER2 negative, "low" Ki-67, and a "low" recurrence risk based on multi-gene-expression assay results if available). Luminal B ("Luminal B-like [HER2 negative]": ER positive, HER2 negative, and at least one of the following: "high" Ki-67, "negative or low" PR, or "high" recurrence risk based on multi-gene-expression assay if available. "Luminal B-like [HER2 positive]": ER positive, HER2 over-expressed or amplified with any Ki-67, and any PR). HER2-enriched (HER2 over-expressed or amplified, HR absent) and TN (negative HR and HER2).

BMI, body mass index; NAC, neoadjuvant chemotherapy; IBC-NST, invasive breast carcinoma of no special Type; pCR, pathological complete response; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer.
TABLE 3 | The univariate relationship between above features with DFS and OS (n = 828).

| Parameter                                | DFS (n = 98) | OS (n = 59) |
|------------------------------------------|--------------|-------------|
|                                          | Events-free  | Events     | p Value | Events-free  | Events     | p Value |
| Age at prognosis (years)                 |              |            |         |              |            |         |
| <40                                      | 76           | 7          | 0.299   | 80           | 3          | 0.181   |
| ≥40                                      | 654          | 91         | 0.045   | 689          | 56         | 0.026   |
| BMI (kg/m²)                              |              |            |         |              |            |         |
| <18.9                                    | 61           | 7          | 0.045   | 62           | 6          | 0.382   |
| 18.9–24.9                                | 323          | 38         | 0.026   | 344          | 17         | 0.382   |
| ≥24.9                                    | 346          | 53         | 0.849   | 363          | 36         | 0.849   |
| Histological type                        |              |            |         |              |            |         |
| IBC-NST                                  | 609          | 90         | 0.849   | 644          | 55         | 0.849   |
| Others                                   | 121          | 8          | 0.721   | 125          | 4          | 0.721   |
| Chemotherapy cycles                      |              |            |         |              |            |         |
| ≤2                                       | 174          | 25         | 0.721   | 184          | 15         | 0.721   |
| 3–5                                      | 377          | 47         | 0.567   | 397          | 27         | 0.567   |
| >5                                       | 179          | 26         | 0.364   | 188          | 17         | 0.364   |
| Chemotherapy regimen                     |              |            |         |              |            |         |
| Taxane-based                             | 77           | 12         | 0.204   | 84           | 5          | 0.204   |
| Anthracycline-based                      | 138          | 12         | 0.026   | 142          | 8          | 0.026   |
| Taxane + anthracycline                   | 514          | 74         | 0.771   | 542          | 46         | 0.771   |
| Anti-HER2 therapy in patients with HER2-positive (n = 261) |              |            |         |              |            |         |
| Yes                                      | 44           | 5          | 0.712   | 47           | 2          | 0.712   |
| No                                       | 181          | 31         | 0.499   | 195          | 17         | 0.499   |
| Clinical tumor stage at diagnosis        |              |            |         |              |            |         |
| T1                                       | 70           | 10         | 0.889   | 72           | 8          | 0.889   |
| T2                                       | 489          | 67         | 0.669   | 519          | 37         | 0.669   |
| T3/T4                                    | 171          | 21         | 0.778   | 178          | 14         | 0.778   |
| Post-NAC tumor size                      |              |            |         |              |            |         |
| <2 cm                                    | 384          | 50         | 0.871   | 404          | 30         | 0.871   |
| 2–5 cm                                   | 311          | 44         | 0.778   | 328          | 27         | 0.778   |
| >5 cm                                    | 35           | 4          | 0.712   | 37           | 2          | 0.712   |
| Clinical nodal status                    |              |            |         |              |            |         |
| Positive                                 | 597          | 78         | 0.256   | 627          | 48         | 0.256   |
| Negative                                 | 132          | 20         | 0.293   | 141          | 11         | 0.293   |
| ER statusa                               |              |            |         |              |            |         |
| Positive                                 | 471          | 55         | 0.377   | 494          | 32         | 0.377   |
| Negative                                 | 259          | 43         | 0.246   | 275          | 27         | 0.246   |
| PR positivity scoreb                     |              |            |         |              |            |         |
| <20%                                     | 449          | 71         | 0.246   | 474          | 46         | 0.246   |
| ≥20%                                     | 281          | 27         | 0.059   | 295          | 13         | 0.059   |
| HER2                                     |              |            |         |              |            |         |
| Positive                                 | 225          | 36         | 0.275   | 242          | 19         | 0.275   |
| Negative                                 | 402          | 45         | 0.862   | 417          | 30         | 0.862   |
| Unknown                                  | 103          | 17         | 0.607   | 110          | 10         | 0.607   |
| Pre-NAC Ki67                             |              |            |         |              |            |         |
| <30%                                     | 227          | 26         | 0.438   | 237          | 16         | 0.438   |
| ≥30%                                     | 503          | 72         | 0.004   | 532          | 43         | 0.004   |
| Post-NAC Ki67                            |              |            |         |              |            |         |
| <30%                                     | 446          | 45         | 0.008   | 467          | 24         | 0.008   |
| ≥30%                                     | 284          | 53         | 0.571   | 302          | 35         | 0.571   |
| Molecular subtypesc                      |              |            |         |              |            |         |
| Luminal A                                | 42           | 1          | 0.335   | 42           | 1          | 0.335   |
| Luminal B                                | 432          | 57         | 0.571   | 456          | 33         | 0.571   |
| HER2-enriched                            | 128          | 20         | 0.113   | 137          | 11         | 0.113   |
| TNBC                                     | 128          | 20         | 0.113   | 134          | 14         | 0.113   |

*Positivity score<1% including negative status.

*Positivity score>20% including negative status.

*Luminal A: (ER and PR positive, HER2 negative, "low" Ki-67, and a "low" recurrence risk based on multi-gene-expression assay results if available). Luminal B ("Luminal B-like [HER2 negative]": ER positive, HER2 negative, and at least one of the following: "high" Ki-67, "negative or low" PR, or "high" recurrence risk based on multi-gene-expression assay if available). Luminal B-like [HER2 positive]": ER positive, HER2 over-expressed or amplified with any Ki-67, and any PR); HER2-enriched (HER2 over-expressed or amplified, HR absent) and TN (negative HR and HER2).

BMI, body mass index; NAC, neoadjuvant chemotherapy; IBC-NST, invasive breast carcinoma of no special Type; pCR, pathological complete response; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer.
FIGURE 2 | Kaplan-Meier curve of survival in patients with pCR status; Luminal A subtype (A, B), Luminal B subtype (C, D), HER2-enriched subtype (E, F), TN breast cancer subtype (G, H). Blue lines: achieving pCR; Red lines: not achieving pCR. The left side of figure represented the relationship between pCR status and DFS. The right side presented the relationship between pCR status and OS. Abbreviations: pCR, pathological complete response; HER2, human epidermal growth factor receptor 2; TN breast cancer, triple negative breast cancer; DFS, disease-free survival; OS, overall survival.
confirmed as invasive carcinoma of NST for 84.4% of patients. All other histological types represented 15.6% of the full cohort. A median of 4 NAC cycles were received (range: 1–9), with NAC classified into three groups: taxane-based (10.7%), anthracycline-based (18.1%) and taxane + anthracycline (71.1%). The average maximum tumor diameters before and after NAC were 3.41 ± 1.651 and 2.26 ± 1.648 cm, respectively. 81.9% patients presented with node-positive status at diagnosis. Finally, 138 subjects (16.7%) who received NAC achieved pCR, a commonly used measurement of NAC efficacy.

We next analyzed IHC biomarkers. Patients with ER positivity made up 59.5% of the cohort. Patients with PR < 20%, or negative status, represented 62.8% of the cohort. Based on strict IHC staining, subjects positive for HER2 accounted for 31.5% of cases 87.6% of all cases had Ki67 expression ≥ 30% before NAC, while only 58.9% of the cohort had ≥30% Ki67 after NAC. 18.8% of HER2-positive patients received anti-HER2 therapy.

Based on biomarker status, patients were categorized into four subtypes: 43 subjects (5.2%) were categorized as Luminal A subtype breast cancer, 489 subjects (59.1%) had a Luminal B breast cancer, 148 subjects (17.9%) had HER2-enriched breast cancer, and 148 cases (17.9%) had TN breast cancer. IHC status and subtypes distribution are shown in Table 1.

Correlation Between Patient Features and Pathological Response to NAC or Survival

We chose pCR as our evaluation criterion of pathological response to NAC. The median follow-up time was 62.00 ± 21.43 months. A associations between patient features and pathological response to NAC or survival were assessed via the Chi-square test ($\chi^2$), with results shown in Table 2. Age, body mass index, maximum tumor diameter before NAC, and HER2 status all had $p$ values > 0.05, indicating no significant influence on prognosis. However, different carcinoma pathology subtypes, maximum diameter after NAC, and nodal status at diagnosis were all significantly associated with pCR, with all $p$ values < 0.05. The IHC biomarkers ER, PR and Ki67 status before and after NAC, all associated with pathological response to NAC. The log-rank test was used for in analysis of different parameters with DFS and OS fully considering the follow-up time (Table 3).
TABLE 5 | The univariate analysis of relation between basic characteristics with ΔKI67% status.

| Parameter | ΔKI67% ≤ –63% (Positive) | ΔKI67% > –63% (Negative) | p Value |
|-----------|--------------------------|--------------------------|---------|
| Age at prognosis (years) | | | |
| <40 | 24 | 59 | 0.105 |
| ≥40 | 283 | 462 | |
| BMI (kg/m²) | | | 0.208 |
| <18.9 | 26 | 42 | |
| 18.9–24.9 | 145 | 216 | |
| >24.9 | 136 | 263 | |
| Histological type | | | <0.001 |
| IBC-NST | 228 | 471 | |
| Others | 79 | 50 | |
| Chemotherapy cycles | | | <0.001 |
| ≤2 | 48 | 151 | |
| 3–5 | 176 | 248 | |
| >5 | 83 | 122 | |
| Chemotherapy regimen | | | <0.001 |
| Taxane-based | 26 | 63 | |
| Anthracycline-based | 81 | 69 | |
| Taxane + anthracycline | 200 | 389 | |
| Anti-HER2 therapy in patients with HER2-positive (n = 261) | | | 0.265 |
| Yes | 22 | 27 | |
| No | 77 | 135 | |
| Clinical tumor stage at diagnosis | | | 0.002 |
| T1 | 21 | 59 | |
| T2 | 196 | 360 | |
| T3/T4 | 90 | 102 | |
| Post-NAC tumor size | | | 0.169 |
| <2 cm | 174 | 260 | |
| 2–5 cm | 120 | 235 | |
| >5 cm | 13 | 26 | |
| Clinical nodal status | | | 0.011 |
| Positive | 237 | 439 | |
| Negative | 70 | 82 | |
| ER status* | | | 0.884 |
| Positive | 196 | 330 | |
| Negative | 111 | 191 | |
| PR positivity score** | | | 0.532 |
| <20% | 197 | 323 | |
| ≥20% | 110 | 198 | |
| HER2 | | | 0.771 |
| Positive | 99 | 162 | |
| Negative | 161 | 286 | |
| Unknown | 47 | 73 | |
| Pre-NAC Ki67 | | | <0.001 |
| <30% | 67 | 186 | |
| ≥30% | 240 | 335 | |
| Post-NAC Ki67 | | | <0.001 |
| <30% | 303 | 188 | |
| ≥30% | 4 | 333 | |
| Molecular subtypes*** | | | <0.001 |
| Luminal A | 2 | 41 | |
| Luminal B | 195 | 294 | |
| HER2-enriched | 51 | 97 | |
| TNBC | 59 | 89 | |

*Positivity score<1% including negative status.
**Positivity score>20% including negative status.
***Luminal A: (ER and PR positive, HER2 negative, "low" Ki-67, and a "low" recurrence risk based on multi-gene-expression assay results if available). Luminal B ("Luminal B-like [HER2 negative]"): ER positive, HER2 negative, and at least one of the following: "high" Ki-67, "negative or low" PR, or "high" recurrence risk based on multi-gene-expression assay if available. "Luminal B-like [HER2 positive]": ER positive, HER2 over-expressed or amplified with any Ki-67, and any PR; HER2-enriched (HER2 over-expressed or amplified, HR absent) and TN (negative HR and HER2). BMI, body mass index; NAC, neoadjuvant chemotherapy; IBC-NST, invasive breast carcinoma of no special Type; pCR, pathological complete response; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer.
For DFS and OS, BMI and Ki67 after NAC both presented \( p \) value < 0.05. Histological subtype further affected both pCR rates and survival status. Each subtype resulted in different pathological responses, as validated by the Chi-square test \( (p < 0.05) \), but there was no obvious change on patient prognosis \( (p = 0.244 > 0.05) \). Rates of pCR across subtypes are shown in Figure 2. The left side of the picture presents the relation between pCR and DFS. The right side of the picture presents the univariate analysis of the relationship between OS and pCR based on the Kaplan-Meier method. We calculated survival rates for patients with each subtype using the Kaplan–Meier method.
pCR status had no impact on DFS outcome for patients with Luminal A ($p = 0.784 > 0.05$, Figure 2A) or Luminal B subtypes ($p = 0.427 > 0.05$, Figure 2B). However, both HER2-enriched and TN breast cancer subtype patients showed a significant association between pathological response to NAC and survival outcome ($p = 0.043 < 0.05$, and $p = 0.042 < 0.05$, respectively, Figures 2C,D). The corresponding Kaplan-Meier curves of pCR with OS outcome for four subtypes are presented in Figures 2E-H. The multivariate Cox analysis for the full cohort is available in Supplementary Table S1. The visual result of univariate analysis displayed via Kaplan-Meier curves is in Supplementary Figure S1.

**Assessment of the Prognostic Efficacy of Ki67 Expression Status Before and After NAC**

The average Ki67 status before NAC was 39.66% ± 22.61%. In comparison, the average value after NAC was 25.00% ± 22.91%. The downward trend of Ki67 before and after NAC is displayed in Figure 3 for the whole cohort (A), Luminal subtype (B), HER2-enriched subtype (C), and TN subtype (D). As shown in the figure, Ki67 change before and after NAC presented a statistical difference ($p < 0.001$) in Luminal subtype. However, in HER2-enriched and TN breast cancer, the difference of Ki67 represented no statistical significance. To evaluate the degree of Ki67 decline, we compared post-NAC and pre-NAC proliferation indices [define post-NAC Ki67 as A, define pre-NAC Ki67 as B, the computational formula was $\Delta Ki67\% = (A - B) ÷ B \times 100\%$, maintaining sign].

We next used ROC curve analysis to determine the optimal cut-off value for $\Delta Ki67\%$. Performing the calculations on SPSS 26.0, we found that $\Delta Ki67\%$ had prognostic efficacy on survival outcomes among the full cohort. Based on the ROC calculation results in all patients, we defined an optimal cut-off of $\Delta Ki67\% \leq −63\%$ ($p < 0.05$). The $\Delta Ki67\%$ cut-off point in the Luminal B subtype was “−63%” ($p = 0.047$). Meanwhile the cut-off point in the TN breast cancer subtype was “−68%” ($p = 0.009$) (Table 4).

Therefore, we defined a reduction of greater than 63% ($\Delta Ki67\% \leq −63\%$) as $\Delta Ki67\%$ positive, and a reduction of less than 63% ($\Delta Ki67\% > −63\%$) as $\Delta Ki67\%$ negative. Positive $\Delta Ki67\%$
status presented a larger magnitude of change for ΔKi67% between pre- and post-NAC, with negative status showing opposite. We used Chi-square tests to evaluate the relationship between demographic and pathological features and ΔKi67% status (Table 5). ΔKi67% -positive patients represented 37.1% of the full cohort. Histological type, number of chemotherapy cycles, type of chemotherapy regimen, clinical nodal status, molecular subtypes, pre- and post-NAC tumor size, and Ki67 all showed statistically significant relationship with ΔKi67% status (p values < 0.05).

In summary, ΔKi67% -positive status related with better survival outcomes. We used the Kaplan-Meier method to affirm the correlation between ΔKi67% status and survival outcomes in each molecular subtype. Survival curves of DFS and OS based on ΔKi67% status for the four subtypes are displayed in Figures 4A–H. Similar to Figure 2, the left side of the figure presents the Kaplan-Meier curves related with DFS and ΔKi67%, with the right side presenting the relationship between OS and ΔKi67%. As shown in the figure, the univariate log-rank test demonstrated ΔKi67% was statistically significantly related to DFS and OS in Luminal B and TN subtype, while it showed no definite effect in Luminal A and HER2-enriched subtypes (all p > 0.05).

Based on the multivariate Cox analysis, ΔKi67% status is a significant independent prognostic predictor of survival outcome regardless of DFS and OS, with DFS-HR = 3.495 (95% CI 1.723–7.088, p = 0.001) and OS-HR = 23.024 (95% CI 3.552–166.64, p = 0.002) for the Luminal B subtype (Table 6, corresponding forest plot in Figure 5).

Not only that, we tentatively continued to explore the subgroups of Luminal B patients based on the HER2-status. In Luminal B patients from our research, who with negative HER2 status were 256 (52.4%). The patients with positive HER2 status were 113 (23.3%). The patients with unknown HER2 status were 120 (24.5%). The Kaplan-Meier curves and multivariate-analysis results of relationship between ΔKi67% and survival outcome were attached in the Supplementary Presentation S1. Based on the statistical calculation, the analytical results of all subdivisions in Luminal B tumors fully supported the statistical significance of ΔKi67% (univariate and multivariate p < 0.05) except the DFS in HER2-positive Luminal B subtypes (univariate and multivariate p > 0.05). Combined, ΔKi67% was confirmed the statistically significant relationship with disease-free and overall survival outcome.

Among patients with TN breast cancer, ΔKi67% status also provided meaningful survival forecasts on DFS (p = 0.023 < 0.05) and OS (p = 0.019 < 0.05) presented in Figure 4. The relationship between ΔKi67% status and survival outcomes in the TN breast cancer subtype was confirmed by multivariate Cox analysis as well, with DFS-HR = 3.354 (95% CI 1.103–10.196, p = 0.033) and OS-HR = 30.774 (95% CI 3.552–266.64, p = 0.002) (Table 7, corresponding forest plot in Figure 6). Two forest plots represented that negative ΔKi67% status is a valid indicator for better prognostics. We further used the Kaplan-Meier method to affirm the correlation between ΔKi67% status and survival outcomes in each molecular subtype. Survival curves based on ΔKi67% status for the four subtypes are displayed in Figure 6. ΔKi67% status shows statistically significant differences in Luminal B and TN breast cancer patients. The NRI value comparing the prognostic capacity between ΔKi67% status and pCR in TN breast cancer subtype was 0.685 (95% CI 0.336–1.029, p < 0.001), also supporting our conclusions. In both Figures 5, 6 ΔKi67% had a statistically significant relationship with prognostic outcome.

**DISCUSSION**

NAC is currently widely applied to shrink tumors and decrease carcinoma volume, allowing patients to preserve breasts or
### TABLE 7 | The multivariate Cox analysis of Δ Ki67% status in NAC-treated TNBC subtype patients.

| Parameter                              | Disease-free survival | Overall survival |
|----------------------------------------|-----------------------|------------------|
|                                        | HR (95% CI)           | p Value          | HR (95% CI)     | p Value          |
|                                        |                       |                  |                  |                  |
| Age at diagnosis (year)                |                       |                  |                  |                  |
| <40                                    | 1.000                 | 0.715            | 1.000            | 0.988            |
| ≥40                                    | 1.628 (0.173–15.308)  |                  |                  |                  |
| BMI (kg/m²)                            |                       |                  |                  |                  |
| <18.5 (underweight)                    | 1.000                 | 0.743            | 1.000            | 0.154            |
| 18.5–24.9                              | 0.462 (0.056–3.807)   | 0.473            | 0.076 (0.006–1.066) | 0.055            |
| >24.9 (overweight)                     | 0.444 (0.055–5.377)   | 0.446            | 0.179 (0.018–1.804) | 0.179            |
| Histological type                      |                       |                  |                  |                  |
| IBC-NST                                | 1.000                 |                  | 1.000            |                  |
| Others                                 | 0.429 (0.080–2.315)   | 0.105            | 1.154 (0.109–12.233) | 0.027            |
| Clinical nodal status at diagnosis     |                       |                  |                  |                  |
| Positive                               | 1.000                 |                  | 1.000            |                  |
| Negative                               | 0.307 (0.074–1.281)   | 0.225            | 1.032 (0.022–0.798) | 0.469            |
| Chemotherapy cycles                    |                       |                  |                  |                  |
| ≤2                                     | 1.000                 |                  | 1.000            |                  |
| 3–5                                    | 1.647 (0.476–5.693)   | 0.431            | 0.879 (0.203–3.811) | 0.863            |
| ≥5                                     | 0.484 (0.089–2.449)   | 0.380            | 0.315 (0.043–2.294) | 0.254            |
| Clinical tumor stage at diagnosis      |                       |                  |                  |                  |
| T1                                     | 1.000                 | 0.378            |                  |                  |
| T2                                     | 2.265 (0.328–15.616)  | 0.407            | 9.973 (0.513–194.007) | 0.129            |
| T3/T4                                  | 0.557 (0.038–8.260)   | 0.671            | 6.405 (0.167–246.220) | 0.319            |
| Post-NAC tumor size                    |                       |                  |                  |                  |
| <2 cm                                  | 1.000                 | 0.462            | 1.000            | 0.405            |
| 2–5 cm                                 | 1.048 (0.369–2.976)   | 0.929            | 0.939 (0.217–4.060) | 0.933            |
| >5 cm                                  | 4.086 (0.435–38.418)  | 0.218            | 5.091 (0.414–62.551) | 0.204            |
| Pre-NAC Ki67                           |                       |                  |                  |                  |
| <30%                                   | 1.000                 | 0.137            | 1.000            | 0.089            |
| ≥30%                                   | 4.442 (0.624–31.635)  | 0.027            | 8.686 (0.722–104.507) | 0.032            |
| Post-NAC Ki67                          |                       |                  |                  |                  |
| <30%                                   | 1.000                 | 0.027            | 1.000            | 0.033            |
| ≥30%                                   | 6.880 (1.238–38.224)  | 1.000            | 7.221 (1.181–44.133) | 0.002            |
| ΔKi67%                                 |                       |                  |                  |                  |
| ≤−63%                                  | 1.000                 |                  | 1.000            |                  |
| >−63%                                  | 3.354 (1.103–10.196)  |                  | 30.774 (3.552–266.644) |                  |

BMI, body mass index; NAC, neoadjuvant chemotherapy; IBC-NST, invasive breast carcinoma of no special type.
become operable [15]. Moreover, the pathological response to NAC is also beneficial for optimizing chemotherapy regimens and predicting relapse possibility and survival outcomes. Patients with pCR to NAC show improved rates of relapse and better survival [33,34].

Many studies have verified correlations between the degree of Ki67 reduction and pathological response to NAC [10,35,36], yet controversies exist regarding the relationships between Ki67 index, pathological response, and survival rates. Some studies only find significant Ki67 proliferation index differences when comparing pre-NAC with post-surgery in Luminal subtypes [37,38] while other studies have demonstrated that Ki67 reduction also plays a role in TN breast cancer [39,40]. While a separate investigation mentioned ΔKi67%, it only discussed its prognostic role and predictive value within 90 Luminal subtype patients with neoadjuvant letrozole-based treatment without classifying it more broadly [20].

In this retrospective real-world study, we analyzed basic demographic and pathological characteristics relative to pCR following to NAC. We confirmed that breast cancer pathological subtype, chemotherapy cycle number, maximum tumor diameter after NAC, nodal state at diagnosis, and Ki67 index pre-NAC and post-NAC all presented statistically significant differences. Furthermore, ER and PR status and molecular subtypes all showed significant effects on pCR rate, as verified in previous studies [41].

As mentioned above, pCR rate ranged from 15 to 20% in previous studies [7–9]. Patients who achieved pCR following NAC represented 16.7% of our cohort, a relatively small portion of the total patients. This implies that pCR status increases the specificity of survival outcome predictions but lowered the sensitivity. Many patients were eliminated in the evaluation system, especially those with the Luminal B subtype who represent the largest portion of all breast cancer patients. This is consistent with previous large trials showing pCR rates to have limited prognostic value in patients with Luminal B subtype [11,12]. ΔKi67% status could help improve this deficiency.

Our study has many strengths. Our fundamental statistical data of post-NAC Ki67 is in accordance with previous research about the relevance of clinical response to NAC and prognostic value [12,24,25]. Subtracting pre- and post-NAC Ki67 is insufficient to account for all situations, therefore we used ΔKi67% as a rational solution. ΔKi67% is an indicator capable of considering the extent of Ki67 changes in all individuals. Since achievement of pCR is not a useful prognostic indicator in the Luminal B subtype, the field currently lacks efficient parameters to predict outcome and assist in clinical decisions makings for these patients [11,12].

ΔKi67% is a useful indicator for more than just Luminal B subtype patients. In patients with TN breast cancer, pCR rate and ΔKi67% status both predicted survival outcome with statistical significance. The multivariate analysis confirmed that ΔKi67% status independently predicted long-term outcomes as well. ΔKi67% status may be capable to aid with NAC regimen modification with pCR status in the TN subtype. In Luminal B subtypes, we made the research based on different HER2-status subgroups. Nearly all results of subgroups support our conclusions regardless of DFS and OS. Except the HER2-positive Luminal B tumors, the DFS

\[ p \text{ value of univariate and multivariate analysis is over 0.05.} \]

Meanwhile, considering the function of ΔKi67% in HER2-enriched subtype, it also prompted that HER2/human epidermal growth factor receptor 2) could influence the predictive efficacy of Ki67. The results enlightened us to collect relative data and dig the thoughts deeper.

This study has inherent limitations. Missing data is a common issue in most retrospective single center studies. Hence, we excluded patients whose information was incomplete or inadequate to be incorporated in the study cohort. The second limitation when using Ki67 staining and assessment is lack of stable measurement results [13,14]. To account for this, we adopted the 'hottest-spot' method and performed pathological assessments strictly following international guidelines to improve reproducibility. In the future, artificial intelligence in precision pathology could dramatically improve this method.

In this study, we demonstrate that ΔKi67% status serves as an independent prognostic factor in Luminal B subtype patients. According to the POETIC clinical phase-3 trial, Ki67 variation in women with operable ER-positive primary breast cancer after preoperative and perioperative aromatase inhibitor (POAI) therapy assisted in deciding further adjuvant endocrine therapy and chemotherapy [19]. This indicates that ΔKi67% could fill the current gap for predicting prognostic outcomes in Luminal B subtype patients and assist in further clinical treatment decisions to help modify further adjuvant regimens.

CONCLUSION
In this study, we validated that the extent of Ki67 change before and after NAC, termed ΔKi67%, associates with patient survival outcomes across subtypes. Our statistical calculations defined a cut-off value for ΔKi67% of (−63%). We confirmed that ΔKi67% status presents an independent prognostic prediction indicator for long-term outcome in Luminal B and TN breast cancer subtypes. As pCR achievement is not a statistically significant predictor for Luminal B subtype patients, ΔKi67% status may fill this clinical vacancy, assisting with measuring efficacy of neoadjuvant therapy and providing data for adjuvant therapy adjustment [42].

DATA AVAILABILITY STATEMENT
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT
This study was allowed by the Ethics Committee of the First Affiliated Hospital of China Medical University. The protocol of this study was approved by the institutional review board (IRB) of the First Affiliated Hospital and was in accordance with the Helsinki Declaration (AF-SOP-07-1.1-01/2019-13). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.
AUTHOR CONTRIBUTIONS

ST analyzed the collected data and wrote the first draft of the manuscript. XF wrote the first draft of the manuscript. XF, SX, PQ, and ZL collected the data and gave assistance on paper revision. YX and QZ were responsible for supervising the whole project and finalizing the manuscript. All authors have read and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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