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Research Article

Keywords: Locally advanced breast cancer; Neo-adjuvant chemotherapy; Residual cancer burden; Hotspot mutations; Metastatic tumor

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11023801/v1>

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Comparative Analysis of Somatic Variants in Paired Pre- and Post-Neoadjuvant Chemotherapy Samples of Locally Advanced Hormone Receptor-Positive Breast Cancer

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Abstract

Background: Neoadjuvant chemotherapy (NACT) is a standard treatment for locally advanced breast cancer (LABC), although hormone receptor-positive (HR+) tumors generally show lower response rates than other molecular subtypes. Patients with residual disease after NACT remain at increased risk of recurrence, underscoring the need to identify molecular determinants of treatment resistance. This study investigated somatic genetic alterations associated with NACT response in HR+ breast cancer stratified by residual cancer burden (RCB).

Methods: A total of 120 HR+ LABC patients treated with NACT were assessed for RCB and classified as good responders (RCB-0/I) or poor responders (RCB-II/III). Targeted next-generation sequencing of ESR1, ESR2, PIK3CA, PTEN, TP53, MAPK1, and AKT1 was performed on matched pre- and post-treatment tumor samples from 20 patients representing different RCB categories. Key alterations were validated by droplet digital PCR in the full cohort. Hotspot mutations in PIK3CA and ESR1 were also evaluated in an independent cohort of 50 metastatic HR+ breast cancer patients.

Results: Pathological complete response was achieved in 26.6% of patients, while 73.3% were poor responders. Recurrent alterations in PIK3CA, TP53, and PTEN were identified. The PIK3CA; p.H1047R hotspot mutation was enriched in pretreatment biopsies from poor responders, suggesting a role in chemotherapy resistance. No mutations were detected in the ESR1 hotspot in the LABC cohort. In contrast, the metastatic cohort harboured ESR1 mutations (p.D538G, p.Y537S) and hotspot PIK3CA variants (p.H1047R, p.E545K, p.E542K).

Conclusions: PIK3CA, particularly p.H1047R, may represent a predictive biomarker of NACT resistance in HR+ breast cancer and warrants validation in larger prospective studies.

Keywords: Locally advanced breast cancer; Neo-adjuvant chemotherapy; Residual cancer burden; Hotspot mutations; Metastatic tumor

Abbreviations:

BC: Breast cancer

ER: Estrogen Receptor

PR: Progesterone Receptor

HER2: Human epidermal growth factor

NACT: Neoadjuvant Chemotherapy

pCR: Pathological complete response

RCB: Residual Cancer Burden

NGS: Next Generation Sequencing

LABC: Locally advanced breast cancer

Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide [1]. Despite advances in screening and treatment, a substantial proportion of patients continue to present with advanced disease, including locally advanced breast cancer (LABC) and metastatic breast cancer. LABC comprises stage III tumors characterized by extension to the chest wall and/or skin (cT4) and/or extensive regional lymph node involvement (cN2–N3) in the absence of distant metastasis [2].

Neoadjuvant chemotherapy (NACT) is the standard treatment for LABC, with the goals of tumor downstaging, improving surgical resection, eradicating micro metastatic disease, and enhancing long-term outcomes [2]. However, pathological response to NACT is highly variable, ranging from pathological complete response (pCR) to substantial residual disease [3,4]. Notably, hormone receptor-positive (HR+) breast cancers exhibit significantly lower pCR rates than HER2-positive and triple-negative subtypes, reflecting inherent differences in tumor biology and chemosensitivity [5].

The somatic mutational landscape of BC provides important insights into mechanisms underlying tumor initiation, progression, therapeutic resistance, and clinical outcome [6]. Intratumoral heterogeneity and clonal evolution contribute to the acquisition of genomic alterations that may influence treatment response and create opportunities for targeted intervention. Elucidating the molecular determinants of resistance is therefore critical for developing personalized therapeutic strategies.

To date, few studies have comprehensively examined somatic alterations associated with NACT response in HR+ breast cancer. In this study, we performed targeted mutational profiling of paired pre-treatment biopsies and post-NACT residual tumors from patients stratified by Residual Cancer Burden (RCB) and Luminal A/Luminal B subtypes. We further compared pretreatment mutational profiles between good and poor responders to identify genomic determinants of chemotherapy resistance. Additionally, an independent cohort of metastatic HR+ breast

cancers was analyzed to characterize molecular alterations associated with disease progression and endocrine therapy exposure.

Material and Methods

Patient cohort: This study included 120 patients with hormone receptor-positive (HR+) LABC, comprising 43 LA and 77 LB tumors. Paired tumor samples, including pre-treatment diagnostic biopsies and corresponding post-NACT residual tumor specimens, were collected. Treatment response was assessed using the RCB calculator developed by the MD Anderson Cancer Center. Patients were categorized into four response groups: RCB-0 (pCR), RCB-I, RCB-II, and RCB-III. In addition, 50 patients with metastatic HR+ breast cancer were enrolled for hotspot mutation analysis. Clinicopathological data, including age, clinical stage, pathological stage, histological subtype, and tumor grade, were retrieved from medical records and analyzed.

Targeted Next-Generation Sequencing: A custom Ion AmpliSeq™ panel (IAD236534_167) comprising 185 amplicons was designed for formalin-fixed paraffin-embedded (FFPE) tissues, covering approximately 98% of the coding regions of seven genes frequently altered in HR-positive breast cancer: *PIK3CA*, *AKT1*, *MAPK1*, *PTEN*, *TP53*, *ESR1*, and *ESR2*. Genomic DNA was extracted using the ReliaPrep™ FFPE gDNA Miniprep System (Promega, A2351) and quantified with the Qubit™ High-Sensitivity dsDNA Assay (Thermo Fisher Scientific, Q32851). Libraries were prepared using the Ion AmpliSeq™ Library Kit Plus (4488990), pooled equimolarly, and subjected to template preparation and enrichment on the Ion OneTouch™ System. Sequencing was performed on the Ion GeneStudio™ S5 platform (Thermo Fisher Scientific). Raw sequencing data were processed using Torrent Suite Software v5.0.4. Reads were aligned to the GRCh38/hg38 reference genome using the Torrent Mapping Alignment Program, and variant calling and annotation were performed with Torrent Variant Caller v5.0 using recommended parameters. Variants were classified according to American College of Medical Genetics and Genomics (ACMG) guidelines; variants of uncertain significance were excluded, and a minimum variant allele frequency threshold of 3% was applied for downstream analyses.

Digital PCR: Selected variants identified through NGS were validated using digital PCR (dPCR) in the entire cohort of 120 LABC cases. The validation cohort included 32 diagnostic biopsies from RCB-0 cases, 48 paired samples from RCB-II cases, and 40 paired samples from RCB-III cases. Pre-designed Absolute Q™ dPCR Assays (Thermo Fisher Scientific) targeting *PIK3CA*; p.E545K, *PIK3CA*; p.H1047R and *PIK3CA*; p.E453K were used. Digital PCR was performed using the QuantStudio™ Absolute Q™ Digital PCR System (Applied Biosystems, A52864). Data analysis was performed using QuantStudio™ Absolute Q™ Digital PCR Software. For each sample, two-dimensional scatter plots were examined, and fluorescence thresholds were established using healthy control samples to distinguish wild-type (VIC™ channel) from mutant (FAM™ channel) droplets.

Metastatic cohort: The cohort comprised 50 patients with HR+ metastatic breast cancer for which hotspot mutation analysis of *PIK3CA* and *ESR1* was performed. For *PIK3CA* hotspot mutation screening, real-time PCR was performed using the commercially available EntroGen *PIK3CA* Mutation Detection Kit containing

FAM/HEX-labeled probes targeting the most common activating mutations, including p.C420R, p.E542K, p.E545K, p.E545A, p.E545G, p.E545D, p.Q546E, p.Q546R, p.H1047Y, p.H1047R, and p.H1047L. For ESR1 hotspot mutation analysis, droplet digital PCR (ddPCR) was performed using commercially available FAM/HEX-labeled assays targeting the clinically relevant mutations p.D538G, p.Y537S, and p.L536R. Data were analyzed using QuantaSoft™ software version 1.4.0 according to the manufacturer's recommendations.

Statistical analysis: Associations between molecular alterations and clinicopathological parameters were evaluated using the chi-square test or Fisher's exact test, as appropriate. All statistical analyses were performed using SPSS software (IBM Corp., Armonk, NY, USA). A two-tailed *P* value of <0.05 was considered statistically significant.

RESULTS

Molecular Subtypes and Clinicopathological Characteristics: A total of 120 LABC patients were classified into LA and LB subtypes based on Ki-67 proliferation index and HER2 status. Overall, 43 of 120 cases (35.8%) belonged to the LA subgroup, whereas 77 cases (64.2%) were classified as LB. The mean age of patients was 50 years. Histologically, most tumors were grade II (47.5%) or grade III (44.1%), while grade I tumors accounted for only 8.3% of cases (*p*=0.007). LA tumors predominantly exhibited grade II morphology (55.8%) and LB tumors showed a predominance of grade III histology (53.2%) (Table:1).

Pathological Response to NACT: All patients received standard NACT comprising four cycles of anthracycline-based therapy followed by four cycles of taxane-based chemotherapy before surgical resection. Post-treatment pathological evaluation demonstrated a pCR; pT0N0 in 32 of 120 patients (26.7%), while 88 patients (73.3%) had residual invasive disease. Residual nodal metastasis was identified in 51.6% of cases following neoadjuvant treatment. Lymph node involvement was observed in 62.7% of LA and 45.4% of LB tumors, although this difference was not statistically significant (*p*=0.342). Among the entire cohort, 48 patients (40.0%) were categorized as RCB class II, indicating moderate residual disease, and 40 patients (33.3%) as RCB class III, representing extensive residual disease. When stratified according to molecular subtype, pCR (RCB-0) was achieved in 18.6% (8/43) of LA patients compared with 31.2% (24/77) of LB patients; however, this difference did not reach statistical significance (*p*=0.302). For subsequent analyses, patients with RCB-0 and RCB-I were grouped as good responders, and those with RCB-II and RCB-III as poor responders.

Estrogen receptor expression: Estrogen receptor (ER) expression was assessed in post-NACT residual tumors using immunohistochemistry and quantified by the Allred scoring system. Among the 88 patients with residual disease, ER positivity was retained in 80 cases (90.9%), whereas 8 cases (9.1%) demonstrated loss of ER expression and were reclassified as ER-negative following NACT, suggesting treatment-associated alterations in HR status (Figure 1). Loss of ER expression was observed in 5.7% of LA and 11.3% of LB tumors (*p*=0.469). Similarly, ER loss occurred in 12.5% of patients with RCB class II and 5.0% with RCB class III (*p*=0.223), indicating no significant association between ER conversion and the extent of residual disease.

Targeted next-generation sequencing (NGS): Targeted genomic profiling was performed in 20 patients with LABC. Paired analyses of diagnostic biopsies and matched post-NACT surgical specimens were done for patients

with RCB class II (n=7) and RCB class III (n=9), while four additional diagnostic biopsies from patients achieving pCR (RCB-0) were analyzed as unpaired samples. A total of 36 pre- and post-NACT libraries were prepared and sequenced across three runs using Ion 520™ chips.

Somatic Variants Across Pre- and Post-NACT Specimens

PIK3CA alterations were identified in 26.3% of pretreatment diagnostic biopsies and 26.6% of post-NACT residual tumors. A total of seven pathogenic variants were detected, including three hotspot mutations (*p.H1047R*, *p.E545K*, and *p.E545G*) and four additional pathogenic variants (*p.E453K*, *p.G914R*, *p.M1004I*, and *p.E365K*). These alterations involved exons 6, 8, 10, 19, and 21 and affected the C2, helical, and kinase domains of the protein. The hotspot mutation *p.E545K*, located within the helical domain, was identified in both pretreatment diagnostic biopsies and post-NACT residual tumors, with frequencies of 26.3% and 6.6%, respectively. In pretreatment biopsies, *p.E545K* was observed in both good (50%) and poor (20%) responders. The remaining *PIK3CA* variants (*p.H1047R*, *p.E545G*, *p.E453K*, *p.G914R*, *p.M1004I*, and *p.E365K*) were detected exclusively in post-treatment residual tumors (Figure 2, Supplementary Table 1).

MAPK1 alterations were infrequent, occurring in only 5% (1/20) of patients. A single pathogenic variant, *p.E322K*, located within the C-terminal docking (CD) domain, was identified in a pretreatment diagnostic biopsy. No pathogenic or likely pathogenic *MAPK1* variants were detected in post-NACT residual tumors.

In *AKT1*, only one pathogenic missense mutation, *p.E17K* (c.49G>A), was identified in exon 3 within the N-terminal pleckstrin homology (PH) domain of *AKT1*. This alteration was detected exclusively in a poor responder and was present in both the pretreatment biopsy and matched post-NACT residual tumor, with frequencies of 5.2% (1/19) and 6.6% (1/15), respectively (Supplementary Table 1). No *AKT1* alterations were identified in good responders.

PTEN alterations were observed exclusively in post-NACT residual tumors, affecting 20% (4/15) of patients. Nucleotide change analysis revealed a predominance of C>T transitions. Six pathogenic variants involving exons 1, 7, and 8 were identified within the conserved C2 domain. These comprised three missense mutations (*p.Q17X*, *p.P246L*, and *p.T375I*) and three truncating variants (*p.Q344Ter*, *p.R406Ter*, and *p.Q434Ter*) (Figure 2, Supplementary Table 1). No *PTEN* alterations were detected in pretreatment diagnostic biopsies.

TP53 was the most frequently altered gene, with mutations detected in 36.8% (7/19) of pretreatment diagnostic biopsies and 20% (3/15) of post-NACT residual tumors. Most pathogenic variants clustered within exons 4–9, corresponding to the DNA-binding domain. A total of eight pathogenic variants were identified, comprising three missense and five truncating mutations. Seven variants (*p.R267W*, *p.R175H*, *p.G245V*, *p.Q104Ter*, *p.R213Ter*, *p.Q331Ter*, and *p.Q167Ter*) were detected in pretreatment samples, whereas only two variants (*p.R267W* and *p.Q144Ter*) persisted in residual tumors (Figure 2, Supplementary Table 1). Variant burden was higher in poor responders than in good responders. In the pretreatment setting, good responders harboured three variants (*p.R267W*, *p.R175H*, and *p.Q104Ter*), while poor responders exhibited six variants (*p.R267W*, *p.R175H*, *p.G245V*, *p.R213Ter*, *p.Q331Ter*, and *p.Q167Ter*).

Validation of somatic variants detected in LABC:

Three PIK3CA variants (p.E545K, p.H1047R and p.E453K) were validated in separate cohort (n=120). Evaluation was performed on patients from RCB0 (n=32), and paired analysis was performed on RCBII (n=48/88) and RCBIII (n=40/88) patients. Ready-to-use primer and probe sets, Absolute QTM dPCR Assay (40X), were acquired from Thermo Fisher Scientific.

The PIK3CA; p.H1047R variant was detected in 30.8% (n=37/120) LABC patients. Overall, it was found in 21.6% (n=26/120) pre-NACT diagnostic biopsies and 22.7% (n=20/88) post-NACT residual tumors (p=0.85). This variant was significantly enriched in diagnostic biopsies from the poor responder group (27.2%; n=24/88) compared with the good responder group (6.2%; n=2/32) (p=0.01). The PIK3CA; p.E545K variant was detected in 37.5% of patients (n=45/120). Mutation frequency was higher in residual tumor (30.6%) than in pre-NACT diagnostic biopsies (25.8%; p=0.44) (Table 2). No significant difference was observed in mutation frequency in the good responder group (21.8%) and the poor responder group (27.2%) (p=0.55). The PIK3CA; p.E453K variant was detected in 11.6% patients (n=14/120). It was found in 4.1% of diagnostic biopsies and 11.3% of post-NACT residual tumors (p=0.04).

Comparison of Somatic Variants Between LA and LB Tumors

The distribution of validated somatic variants was further examined according to intrinsic molecular subtype. *PIK3CA* (p.H1047R and p.E545K) variants were more prevalent in LB tumors compared with LA tumors (Figure 3). For these variants, mutation frequencies were generally higher in post-NACT residual tumors than in the corresponding pretreatment diagnostic biopsies, suggesting enrichment of resistant subclones following chemotherapy.

No significant difference in the frequency of the *PIK3CA*; p.E453K variant was observed between LA and LB subgroups (p=1.0). However, following NACT, the prevalence of this variant increased from 2.3% in diagnostic biopsies to 14.2% in residual tumors in the LA subgroup and from 5.1% to 9.4% in the LB subgroup, indicating possible chemotherapy-mediated clonal selection.

Correlation with Clinicopathological Characteristics

Statistical analysis demonstrated that the *PIK3CA*; p.H1047R hotspot mutation was significantly associated with residual cancer burden (RCB) categories in pretreatment diagnostic biopsies (p=0.001) and with lymph node metastasis (p=0.006). No significant associations were observed between *PIK3CA* mutation status and other clinicopathological parameters, including tumor grade, overall RCB class, intrinsic molecular subtype, or Ki-67 proliferation index. At the time of analysis, all patients in the LABC cohort were alive, precluding meaningful survival analysis and assessment of the prognostic impact of these somatic variants.

Hotspot ESR1 and PIK3CA Variants in Metastatic/Relapsed ER-Positive Breast Cancer

Hotspot variants of *ESR1* and *PIK3CA* were validated in an independent cohort of 50 patients with metastatic or relapsed ER+ BC. Molecular subtyping demonstrated that 40% (n=20) of tumors belonged to the LA subgroup, while 60% (n=30) were classified as LB. The mean patient age was 51 years. Patients were further stratified by metastasis site: brain (46%, 23/50), bone (24%, 12/50), liver (14%, 7/50), lung (10%, 5/50), and 1 patient each with orbital, rectal, and gastric metastases.

ESR1; *p.D538G* variant was detected in 8% (4/50) of patients, including 2 with brain metastases, 1 with lung metastases, and 1 with rectal metastases. The median overall survival (OS) of patients harboring the *p.D538G* mutation was 22 months, although this did not reach statistical significance ($p=0.69$). The *p.Y537S* mutation was identified in a single patient with brain metastasis (Table 3), whereas the *p.L536R* variant was not detected in the study cohort.

PIK3CA Real-time PCR analysis was performed on all 50 metastatic cases; however, successful results were obtained in 38 patients. Overall, *PIK3CA* mutations were identified in 34.2% of evaluable patients. Three hotspot variants were detected: *p.H1047R* (21%), *p.E542K* (7.8%), and *p.E545K* (5.2%), with *p.H1047R* being the predominant alteration.

Kaplan–Meier survival analysis demonstrated median OS of 10 months for patients harboring the *PIK3CA*; *p.H1047R* mutation ($p=0.107$), 7 months for those with *p.E545K* mutations ($p=0.142$), and 2.5 months for those with *p.E542K* mutations ($p=0.146$) (Figure 4, Table 3). Although none of these associations reached statistical significance, the findings suggest potential heterogeneity in clinical outcomes according to specific *PIK3CA* hotspot variants.

Discussion

Most NACT studies in breast cancer have focused on response rates, RCB, and survival outcomes [1, 2]. However, in view of substantial intratumoral heterogeneity, with multiple co-existing subclones, somatic alterations in paired pre- and post-NACT specimens may provide additional insights into mechanisms of treatment response, clonal evolution, and therapeutic resistance. In the present study, we evaluated the somatic mutational landscape of HR+ LABC across different RCB classes and molecular subtypes and further assessed the hotspot *PIK3CA* and *ESR1* mutations in an independent metastatic cohort.

In our LABC cohort, LB tumors predominated (64.1%) over LA tumors (35.8%), consistent with recent data from North India reported by Somal et al. [3], although earlier studies described a higher prevalence of LA disease [4]. We also observed loss of ER expression in 9% of post-NACT residual tumors, comparable to the 11.7% reported by Coiro et al. [5], underscoring the importance of reassessing biomarker status after neoadjuvant therapy to guide adjuvant treatment decisions. Only 26.6% of patients achieved pathological complete response (RCB-0), while 73.4% exhibited substantial residual disease (RCB-II/III). Although LB tumors demonstrated a modestly higher pCR rate than LA tumors (31.1% vs. 18.6%), both subtypes showed limited overall chemosensitivity. Previous studies have noted that the highest pCR rates are in HER2, followed by TNBC and LB, while LA shows the lowest chemosensitivity and pCR across all four molecular subtypes [6]. These findings emphasize the need for predictive biomarkers and alternative therapeutic strategies to improve outcomes in HR-positive breast cancer.

Targeted NGS of paired samples identified *TP53* (50%) and *PIK3CA* (45%) as the most frequently altered genes, followed by *PTEN* (20%), *AKT1* (10%), and *MAPK1* (5%). No pathogenic variants were detected in *ESR1* or *ESR2*, consistent with previous reports indicating that these mutations are uncommon in treatment-naïve tumors and predominantly emerge after endocrine therapy [7, 8].

Previous studies have reported substantial variability in the prevalence of *PIK3CA*, *TP53*, and *PTEN* mutations among HR-positive breast cancers worldwide [9–11]. The slightly higher mutation frequencies observed in our

cohort may, in part, be attributable to the relatively small sample size of the NGS discovery set. Nevertheless, the frequency of *PIK3CA* alterations in our study was comparable to that reported by Xiao et al. [9]. Validation in a larger cohort demonstrated significant enrichment of the *PIK3CA*; *p.H1047R* hotspot mutation in pretreatment biopsies from poor responders, suggesting its potential utility as a predictive biomarker for NACT response. Importantly, these findings highlight that individual *PIK3CA* variants may differ in their biological and clinical implications.

AKT1, a key downstream effector of the PI3K signaling pathway, harbored the canonical *E17K* mutation in exon 3 consistent with previous reports [11, 12]. Notably, this alteration was detected exclusively in poor responders and persisted in both pretreatment and post-NACT specimens, suggesting a potential association with NACT resistance.

Previous studies have reported *TP53* mutation frequencies of 12–18% in Luminal Breast cancers, with substantially higher rates in HER2-positive and triple-negative subtypes [13, 14]. Notably, *PIK3CA*, *TP53*, and *PTEN* alterations were more common in LB tumors than in LA tumors, consistent with findings from the TCGA dataset. Likewise, Choi et al. [15] reported increased frequencies of *PIK3CA* and *TP53* mutations in LB breast cancers, suggesting that the greater mutational burden of this subtype may underlie its more aggressive clinical behavior and comparatively poorer therapeutic response. Furthermore, *PIK3CA*, *AKT1*, and *PTEN* alterations were enriched in post-NACT residual tumors relative to pretreatment diagnostic biopsies. This observation reflects chemotherapy-driven selection and expansion of pre-existing resistant subclones rather than the de novo acquisition of mutations during treatment [16]. Supporting this interpretation, Venizelos et al. [17] demonstrated that clones emerging in residual disease after therapy are often detectable in baseline tumors at frequencies below the limits of conventional sequencing approaches.

We further evaluated hotspot *PIK3CA* and *ESR1* variants in a cohort of metastatic HR-positive breast cancer patients. Consistent with the findings of Martinez-Saez et al. [18], approximately 80% of *PIK3CA* mutations occur in exons 9 and 20. In our cohort, *PIK3CA* mutations were identified in 34.2% of patients. Although this frequency is lower than that reported in some previous studies [19] [20], it is comparable to that described by Dieci et al. [21]. The three canonical hotspot mutations detected were *p.H1047R* (21%), *p.E542K* (7.8%), and *p.E545K* (5.2%). These gain-of-function alterations in the p110 α catalytic subunit are established drivers of PI3K pathway activation, promoting tumor cell proliferation, survival, and oncogenic progression [18].

In contrast, *ESR1* mutations are rare in primary breast cancers but commonly emerge in metastatic disease following endocrine therapy, with reported frequencies ranging from 10% to 50% [22, 23]. In our metastatic cohort, *ESR1* mutations were detected in 8% of patients. Among the hotspot variants analyzed, only *p.D538G* (8%) and *p.Y537S* (2%) were identified, whereas *p.L536R* was absent. Both *p.D538G* and *p.Y537S* reside within the ligand-binding domain of the estrogen receptor and confer ligand-independent receptor activation, thereby driving endocrine resistance. The prevalence of *ESR1* mutations also varies according to metastatic site, with higher frequencies reported in liver, bone, and soft-tissue metastases and lower rates in brain metastases [22]. The predominance of brain metastases in our cohort may therefore partly account for the relatively low observed frequency of *ESR1* mutation.

Our findings demonstrate distinct molecular features of Luminal A and Luminal B HR-positive breast cancers, as well as dynamic somatic alterations following neoadjuvant therapy. The association of PIK3CA; *p.H1047R* with poor pathological response highlights its potential as a predictive biomarker for treatment stratification. Limitations include a restricted NGS panel, a small discovery cohort, and the lack of survival analysis due to limited follow-up. As HR-positive breast cancers may recur years after treatment, larger prospective studies with longer follow-up are needed to validate these findings.

Statement and declaration:

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Post-Graduation Medical Education and Research (PGIMER) No. **IEC-INT/2022/PhD-779**.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Informed consent for publication was provided by the participant(s)

Conflict of Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding Statement

We would like to thank the **Indian Council of Medical Education and Research (ICMR)** for **providing fellowship to Ms. Anjali Chadda**.

Data Availability

Data available on request from the authors.

Acknowledgments

We would like to thank **Indian Council of Medical Education and Research (ICMR)** for providing fellowship to Ms. Anjali Chadda.

Declaration of generative AI use

The authors report generative AI was not used in their research or preparation of this manuscript.

Author Contributions

All authors contributed to the study conception and design. **Amanjit Bal:** Conceptualization, Writing – review and editing, Supervision. Material preparation, data collection, and analysis were performed by **Anjali Chadda**. The first draft of the manuscript was written by Anjali Chadda, and all authors (**SK, SI, IL, SB, AS**) commented on earlier versions. All authors read and approved the final manuscript.

Supplementary information

Supplementary Table 1: Pathogenic variants detected in coding region of PIK3CA, AKT1, TP53, MAPK1 and PTEN gene in LABC Patients.

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Figure Legends:

Figure 1 Representative photomicrographs demonstrating (A) retained high ER expression in post-NACT residual tumor, (B) complete loss of ER expression following neoadjuvant chemotherapy, and (C) distribution of ER expression across the overall LABC cohort, Luminal A and Luminal B subgroups, and RCB classes II and III

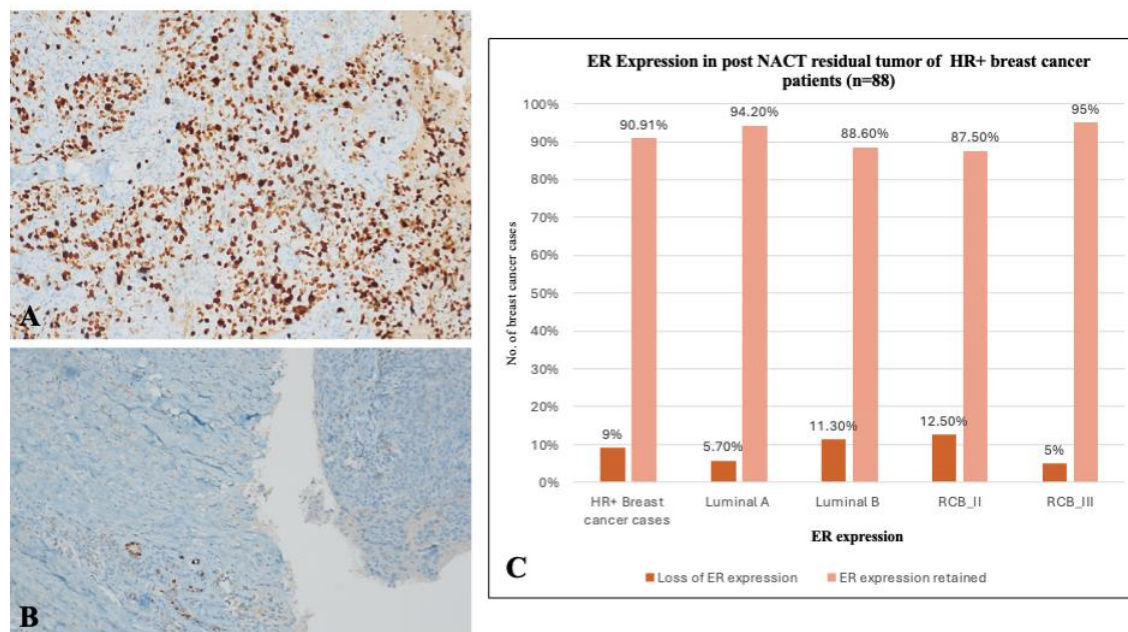


Figure 2 Spectrum of somatic variants across pre- and post-NACT Luminal A breast cancer. (A) Oncoplot illustrating the distribution and frequency of somatic variants in paired pre-treatment diagnostic biopsies and post-NACT residual tumors from patients with HR positive locally advanced breast cancer (LABC). (B) Lollipop plot depicting the distribution of *PIK3CA* variants identified in pretreatment diagnostic biopsies. (C) Lollipop plot showing *PIK3CA* variants detected in post-NACT residual tumors. (D) Lollipop plot showing *TP53* variants in pretreatment diagnostic biopsies. (E) Distribution of *TP53* variants in post-NACT residual tumors. (F) Distribution of *PTEN* variants identified in post-NACT residual tumors. Protein domains are indicated below each lollipop plot. Abbreviations: NACT, neoadjuvant chemotherapy; LABC, locally advanced breast cancer; PH, pleckstrin homology; TAD, transactivation domain; RBD, Ras-binding domain.

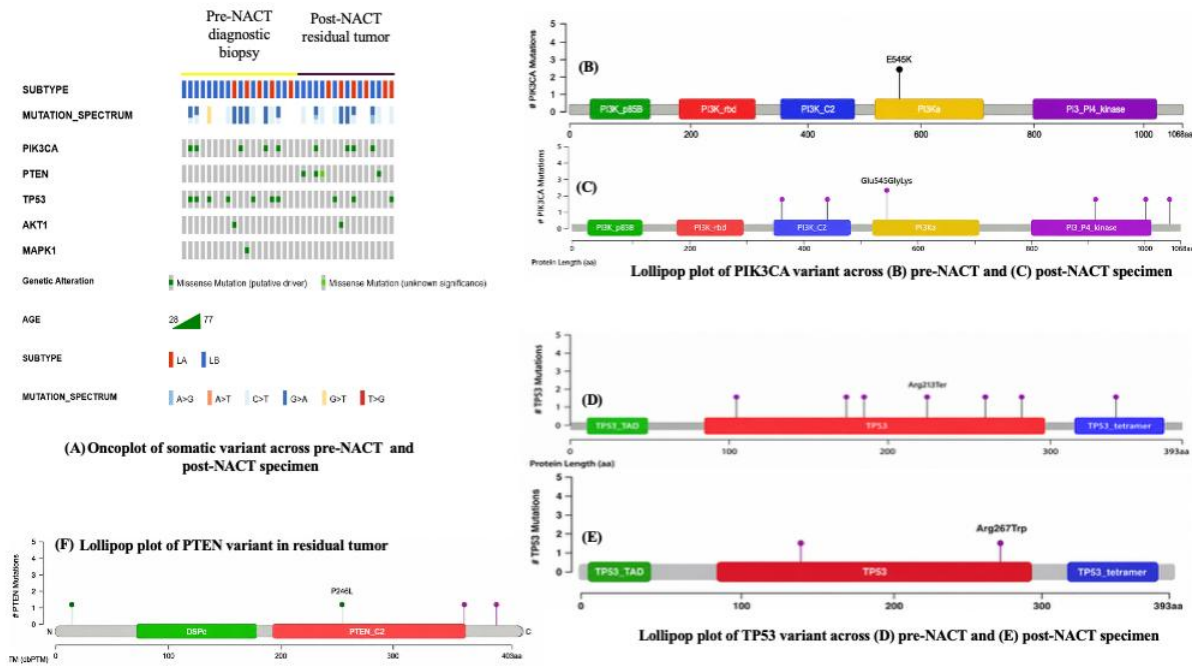


Figure 3 Distribution of validated *PIK3CA* variant frequencies across Luminal A and Luminal B breast cancer subgroups. The graph depicts the prevalence of *PIK3CA* (*p.H1047R*, *p.E545K*, and *p.E453K*) variants in the two molecular subtypes, highlighting the greater enrichment of *PIK3CA* alterations in Luminal B tumors.

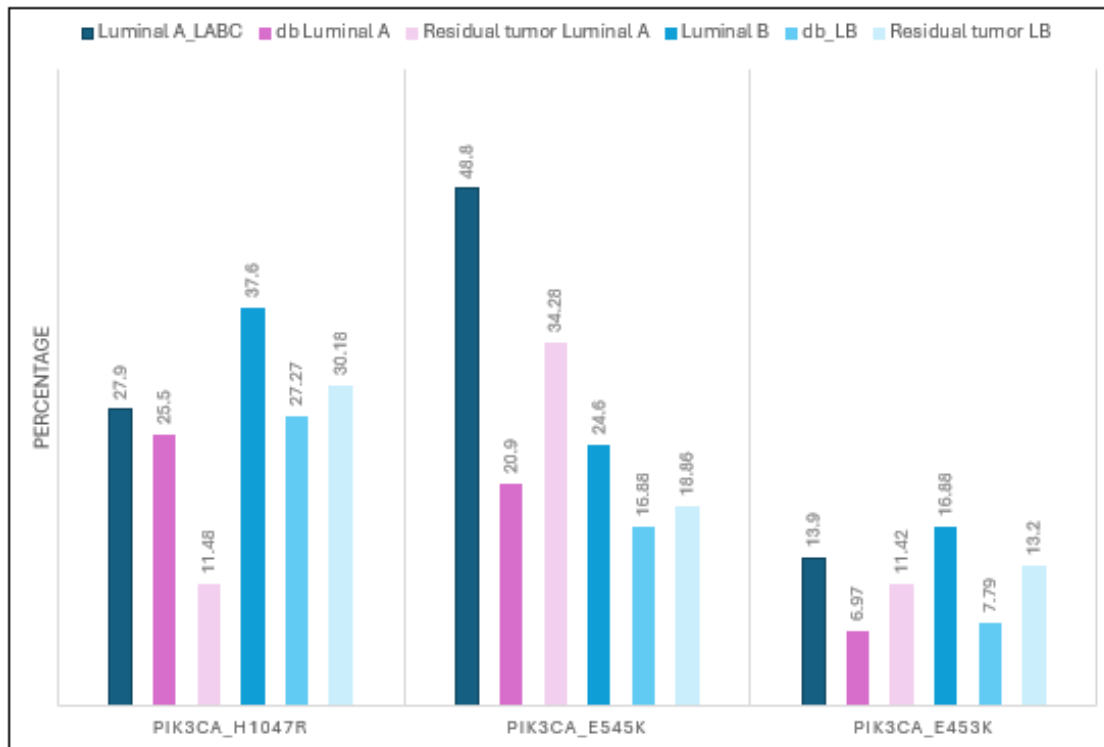


Figure 4 Representative droplet digital PCR (ddPCR) two-dimensional fluorescence plots demonstrating detection of (A) *ESR1*; *p.D538G* and (B) *ESR1*; *p.Y537S* hotspot mutations in metastatic HR-positive breast cancer. Kaplan-Meier survival curves illustrating overall survival according to mutation status for (C) *ESR1*; *p.D538G*, (D) *PIK3CA*; *p.H1047R*, (E) *PIK3CA*; *p.E545K*, and (F) *PIK3CA*; *p.E542K*. Survival estimates are presented as median overall survival from the time of diagnosis of metastatic disease.

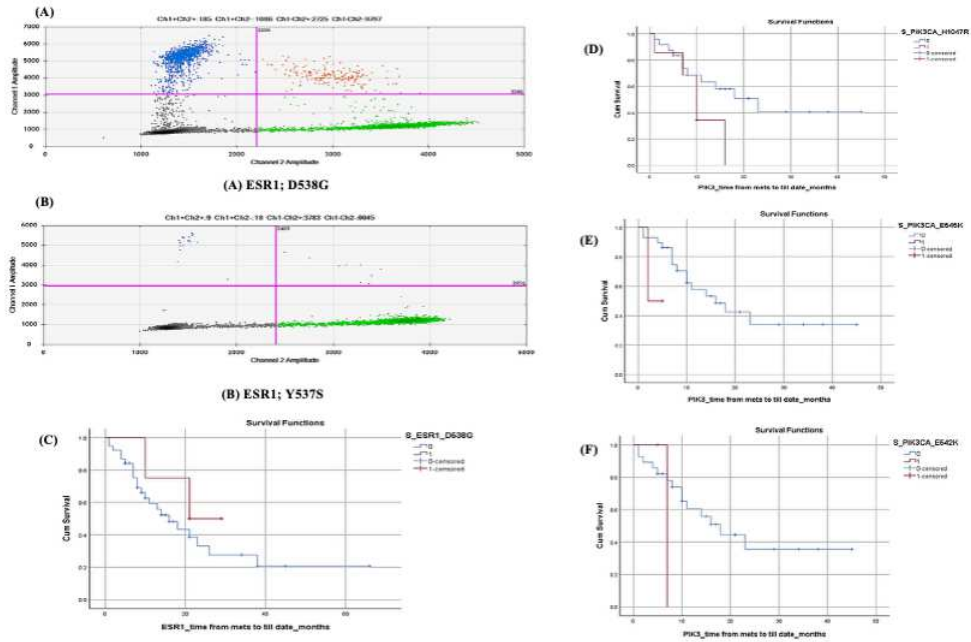


Table 1 Clinicopathological characteristics of HR Positive LABC Patients			
Characteristics		No. of patients (n)	Percentage
Age			
	<35	7	5.8%
	35-55	75	62.5%
	>55	38	31.6%
Mean age	50.77		
Molecular subtype			
	LA	43	35.8%
	LB	77	64.1%
Her2 Status			
	Positive	39	32.5%
	Negative	75	62.5%
	Unknown	6	5%
KI67 expression			
	<14%	53	44.1%
	>14%	63	52.5%

	Unknown	4	3.3%
Tumor grade_Diagnostic biopsy			
	I	10	8.3%
	II	57	47.5%
	III	53	44.1%
Residual Cancer Burden Class			
	RCB0	32	26.6%
	RCBII	48	40%
	RCBIII	40	33.3%
Tumor grade_Post NACT Residual tumor			
	I	9	11.25%
	II	40	50%
	III	31	38.75%
Tumor Size			
	< 2 cm	19	21.5%
	2-5 cm	41	46.5%
	> 5 cm	28	31.8%
Lymph-node metastasis			
	Positive	62	51.6%
	Negative	58	48.3%

Table 2 Pathogenic mutations of PIK3CA, MAPK1 and PTEN gene in LABC Patients											
Gene	Start position	EXON	Nucleotide Change	Effect on Protein	Frequency in LABC patients (n=120)	LA Subgroup (n=43)	LB Subgroup (n=77)	Pre-NACT Diagnostic biopsy (n=120)	Post-NACT residual tumor (n=88)	Diagnostic biopsy_Good responder group (n=32)	Diagnostic biopsy_Poor responder group (n=88)
PIK3CA	Chr3:179210291	8	c.1357G>A	p.Glu453Lys	11.6%(n=14/120)	11.6%(n=5/43)	11.6%(n=9/77)	4.1%(n=5/120)	11.3%(n=10/88)	3.1%(n=1/32)	4.5%(n=4/88)
PIK3CA	Chr3:179218303	10	c.1633G>A	p.Glu545Lys	37.5%(n=45/120)	30.2%(n=13/43)	41.5%(n=32/77)	25.8%(n=31/120)	30.6%(n=27/88)	21.8%(n=7/32)	27.2%(n=24/88)

PIK3CA	Chr3:179234297	21	c.3140A>G	p.His1047Arg	30.8%(n=37/120)	23.2%(n=10/43)	35%(n=27/77)	21.6%(n=26/120)	22.7%(n=20/88)	6.2%(n=2/32)	27.2%(n=24/88)
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Table 3 Depicting clinic-pathological data of metastatic cohort of ER positive tumor (N=50)										
Variant	ESR1_D538G		ESR1_Y537S		PIK3CA_H1047R		PIK3CA_E545K		PIK3CA_E542K	
Mutation	Present	Absent	Present	Absent	Present	Absent	Present	Absent	Present	Absent
No. of patients	8% (n=4/50)	92% (46/50)	2% (n=1/50)	98% (49/50)	21% (n=8/38)	79% (30/38)	5.2% (2/38)	94.7% (36/38)	7.8% (3/38)	92.1% (35/38)
Mean Age (year)	58.25	51.43	57	51.87	60	48.66	54	50.88	53.66	50.82
Luminal A	2	18	0	20	3	12	0	15	1	14
Luminal B	2	28	1	29	5	18	2	21	2	21
Live	2	16	1	17	2	13	1	14	1	14

Dead	2	22	0	24	5	11	1	15	2	14
Mean Survival after metastasis (months)	20.25	14.92	29	15.09	29.5	15	3.5	14.20	5.66	14.28
Lost to follow up	0	8	0	8	1	6	0	7	0	7

Supplementary Files

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