

ORIGINAL Article

Immunohistochemical Concordance of ER, PR, HER2, and p63 Between Frozen-Section Tissue Microarray and Paraffin-Embedded Biopsy in Treatment-Naive Breast Carcinoma: A Prospective Pilot Study

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ABSTRACT

Rapid biomarker assessment on frozen tissue could shorten the time to initiating targeted breast-cancer therapy, but the effect of the freezing process on antigenicity and section quality is incompletely characterized, particularly on the tissue microarray (TMA) format. In this pilot study, we compared estrogen receptor (ER), progesterone receptor (PR), HER2, and p63 immunohistochemistry (IHC) performed on frozen-section TMA cores of treatment-naive breast carcinoma with IHC on the corresponding paraffin-embedded biopsy, and documented section quality and freezing artefacts that may confound the interpretation of immunostaining.

Thirty-five consecutive treatment-naive breast carcinomas were enrolled over one year at our tertiary care cancer centre. Nine were excluded because the TMA cores were thin and could not withstand further processing. ER, PR, HER2, and p63 IHC were done on frozen-section TMA cores and compared with biopsy IHC of the same site for all the remaining 26 cases. Concordance, sensitivity, specificity, and

Cohen's kappa were calculated; section quality and artefacts were graded and correlated with discordance.

Paired frozen-biopsy IHC was evaluable in 22/26 (84.6%) cases. ER and PR showed 100% concordance ($\kappa=1.00$). HER2 showed 77.3% exact four-tier concordance ($\kappa=0.67$; quadratic-weighted $\kappa=0.90$); on a binary cut, sensitivity for a biopsy-positive/equivocal result was 50.0%, while specificity was 100%. p63 was negative in all 22 evaluable frozen-section cases and matched biopsy in all 6 cases where paired biopsy p63 was available (6/6, 100%), confirming that the tumor was invasive. Freezing artefact (66.7%) was the commonest technical problem and correlated significantly with poorer section quality ($p=0.03$).

This pilot study indicates that frozen-section TMA-based IHC for ER and PR is highly reliable; HER2 interpretation on frozen tissue is directionally accurate but should still be anchored to ISH for any non-3+/non-0 result before it is used to guide therapy. Larger, adequately powered validation studies are warranted before routine clinical application.

Key words

Frozen section, tissue microarray, Immunohistochemistry, breast carcinoma; estrogen receptor; HER2, freezing artefact.

Abbreviations

American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP); Estrogen receptor (ER); Formalin-fixed, paraffin-embedded (FFPE); Hematoxylin and eosin (H&E); In situ hybridization (ISH); Global Cancer Observatory (GLOBOCAN); Human epidermal growth factor receptor 2 (HER2); Institutional Ethics Committee (IEC); Immunohistochemistry (IHC); Negative predictive value (NPV); No special type (NST); Positive predictive value (PPV); Progesterone receptor (PR); Ready-to-use (RTU); Statistical Package for the Social Sciences (SPSS); Tissue microarray (TMA).

INTRODUCTION

Breast carcinoma is the most common malignancy among women worldwide and in India. GLOBOCAN 2022 estimates place India third globally in incidence and first in breast-cancer mortality¹. Immunohistochemical (IHC) assessment of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) is mandatory for every newly diagnosed invasive breast carcinoma, since these markers determine eligibility for endocrine and anti-HER2 therapy. Indian series have repeatedly reported lower rates of hormone-receptor positivity than Western cohorts, underscoring the value of locally generated concordance data rather than extrapolation from Western literature^{2,3}.

ER, PR, and HER2 are usually tested on the paraffin-embedded biopsy prior to surgery, which guides treatment. But biopsy has two inherent limitations: the tissue may not be sufficient for the complete panel or repeat testing, and a single small biopsy may not reflect the whole tumor, so its receptor result can differ from that of the resected tumor, which is reported in a significant number of cases, especially for HER2⁴. Intraoperative frozen section is already routinely used for margin assessment and nodal staging in breast surgery and extending this to a rapid receptor and myoepithelial-marker panel could shorten the interval to a definitive biomarker report⁵. However, cryosectioning subject tissue to rapid temperature change, ice-crystal formation, and mechanical distortion, which can

degrade antigenicity and introduce artefact. Whether a frozen-section panel reproduces the biopsy result, and how section quality influences that reproducibility, is incompletely characterized on the tissue microarray (TMA), which is more vulnerable to core fragmentation, core exhaustion and non-representative sampling than a conventional whole-section frozen block⁶.

We therefore undertook this prospective pilot study to evaluate ER, PR, HER2, and p63 IHC on frozen-section TMA cores of treatment-naive invasive breast carcinoma, comparing results with IHC on paraffin-embedded biopsy of the same site, and systematically documenting section quality and artefacts as an attempt to expedite biomarker reporting.

MATERIALS AND METHODS

Study design and setting

This prospective pilot study was carried out in the Department of Oncopathology over one year, in line with the Declaration of Helsinki⁷ and after approval by the institutional ethics committee (approval number: IEC/HBCH/79/25), which granted a waiver of informed consent, as the study does not intend to perform additional procedures on participants or have any effect on their clinical management. As this was a pilot study meant to gather preliminary data, no formal sample-size calculation was done beforehand. All eligible cases during the study period were included, and our final sample size was 22 evaluable pairs, which is still larger than the commonly used minimum of 12 per group suggested for pilot studies⁸.

Scoring and blinding

Frozen-section immunostained slides were scored by a pathologist blinded to the paired biopsy IHC result, and biopsy slides were scored blinded to the frozen-section result. Scoring was not independently duplicated by a second observer, so an inter-observer agreement figure could not be calculated; this is noted as a limitation.

Case selection

Consecutive treatment-naive (no prior chemotherapy or hormone therapy) female patients having lumpectomy for invasive breast cancer, with a prior core-biopsy diagnosis, were enrolled. 35 cases were accrued at first; 9 (25.7%) were excluded because the TMA cores were thin and underwent fragmentation

when subjected to further processing, leaving 26 cases (74.3%) as the final study group.

Tissue microarray construction and frozen sectioning

The representative tumor area was identified in the lumpectomy specimen during gross examination. Cores were taken, placed in optimal cutting temperature compound, snap-frozen, and arranged onto a microarray block, adapting the method described by Kononen et al.⁶. Sections of 4–5 µm thickness were cut on a cryostat and subjected to hematoxylin and eosin (H&E) staining for assessment of section quality and freezing artefact.

ER, PR, HER2, and p63 IHC were done on the frozen-section TMA cores using a Ventana BenchMark GX automated platform with ready-to-use antibodies for ER (clone SP1, standard (STD32) cell conditioning), PR (clone 1E2, standard (STD32) cell conditioning), and HER2 (clone 4B5, mild cell conditioning); p63 (clone 4A4, standard (STD32) cell conditioning) was used as a concentrated antibody at 1:300 dilution. ER and PR were scored by the Allred method (proportion 0–5 plus intensity 0–3, total 0–8), with a score of 3 or more counted as positive, following ASCO/CAP guidelines^{9,10}. HER2 was scored 0, 1+, 2+, or 3+ per 2018 ASCO/CAP criteria: 0/1+ negative, 2+ equivocal, and 3+ positive¹¹. p63 highlights the myoepithelial cells in the breast parenchyma but is lost in the invasive tumor. It is interpreted as positive or negative and confirms the invasive phenotype of the tumor. It is considered more reliable than calponin or smooth-muscle myosin heavy chain¹².

Assessment of section quality and artefact

For each case, we recorded two features: section quality (good, average, or poor) and artefact severity (mild, moderate, or severe), along with the artefact type (freezing/ice-crystal, rolling, or folding). Cases in which the core was exhausted before sectioning, or the sampled core was non-representative, were excluded from the paired IHC concordance analysis; however, section quality and artefact, where assessable, were still recorded.

Statistical analysis

Statistical analysis was done using SPSS (Statistical Package for the Social Sciences), version 21.0. Concordance was expressed as a percentage and as Cohen's kappa (κ), with 0.81–1.00, 0.61–0.80, and 0.41–0.60 read as almost-perfect, substantial, and

moderate agreement. HER2 scores form a natural order ($0 < 1+ < 2+ < 3+$), so linear and quadratic weighted kappa were also calculated, which give partial credit for a close disagreement, such as 1+ read as 2+, rather than treating every disagreement as equally wrong. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using biopsy as the reference standard. Associations between section quality/artefact grade and IHC discordance were tested by Fisher's exact test, and between section quality and artefact severity by the chi-square test. A two-sided p value < 0.05 was considered significant.

RESULTS

Cohort characteristics

Of 35 cases accrued, 9 (25.7%) were excluded because the TMA cores were thin and underwent fragmentation when subjected to further processing, leaving 26 cases for study. All patients were female, with a mean age of 51.2 ± 10.3 years (range 31–74 years). The tumor was left-sided in 14 (53.8%) and right-sided in 12 (46.2%) patients; all underwent lumpectomy, and all tumors were reported as Invasive carcinoma of no special type (ductal). 11 tumors (42.3%) were histological grade 2 and 15 (57.7%) were grade 3.

Evaluable cases

Among the 26 cases, a complete, evaluable, paired panel of ER, PR, HER2, and p63 IHC was obtained on the frozen-section TMA core in 22 (84.6%). In the remaining 4 cases (15.4%), the frozen-section IHC panel could not be evaluated for any marker: in 2 cases (7.7%) the TMA core was completely exhausted before immunostaining could be performed, and in 2 cases (7.7%) the core, although of adequate quantity, did not contain a representative sample of tumor. These four cases were excluded from the paired concordance analysis.

Section quality and artefact

Quality and artefact could be evaluated in 24/26 (92.3%) cases. Overall section quality was good in 10 (41.7%), average in 8 (33.3%), and poor in 6 (25.0%) of the 24 assessable sections. Artefact was mild in 6 (25.0%), moderate in 10 (41.7%), and severe in 8 (33.3%). Freezing/ice-crystal artefact accounted for the great majority of technical problems: freezing artefact alone was the predominant abnormality in 16

(66.7%) sections, discrete ice-crystal artefact in 5 (20.8%), tissue rolling in 2 (8.3%), and tissue folding in 1 (4.2%). Poor section quality was significantly associated with severe artefact grade ($\chi^2=11.04$, $p=0.03$), supporting the internal consistency of the two grading parameters.

ER and PR concordance

Paired frozen-section and biopsy ER and PR results were available in 22/26 (84.6%) cases. ER was positive (Allred score ≥ 3) in 16/22 (72.7%) cases on both frozen section and biopsy, and negative in the remaining 6/22 (27.3%) on both, giving 100% concordance ($\kappa=1.00$; sensitivity, specificity, PPV, and NPV all 100%). PR showed an identical pattern, with 17/22 (77.3%) positive and 5/22 (22.7%) negative concordant on both specimen types (100% concordance, $\kappa=1.00$) (**Table 1**).

HER2 concordance

Exact four-tier HER2 concordance (0/1+/2+/3+) was 77.3% (17/22; $\kappa=0.67$), improving to $\kappa=0.80$ (linear-weighted) and $\kappa=0.90$ (quadratic-weighted), since every discordant pair differed by only one category. On a binary cut (0/1+ negative versus 2+/3+)

positive/equivocal), concordance was 81.8% (18/22; $\kappa=0.56$); sensitivity for a biopsy-positive/equivocal result was 50.0%, with specificity, PPV, and NPV of 100%, 100%, and 77.8%. All five discordant cases scored lower on frozen section than biopsy — four were 1+ versus 2+, one was 0 versus 1+ (**Table 2**); none crossed the negative/positive extremes. All five biopsy-2+ cases were reflexed to ISH and were non-amplified, concordant with frozen section in every instance. Neither poor quality nor severe artefact predicted HER2 discordance (Fisher's exact, $p=0.59$ and $p=1.00$).

p63 concordance

p63 was evaluable on frozen section in all 22 cases and was negative in the tumor cells in 22/22 (100%), consistent with the invasive phenotype. It is important to note that p63 is not a part of the routine breast IHC panel at our institute and is used in cases of diagnostic dilemma between the invasive tumor and in situ component. Therefore, paired biopsy p63 was performed in only 6 of these 22 cases, and it was negative in all 6 cases (100% concordance). A representative case showing ER, PR, HER2 and p63 staining is illustrated in **Figure 1**.

Table 1. Concordance between frozen section and biopsy immunohistochemistry (paired evaluable cases)

IHC marker	n	Concordant, n (%)	Sensitivity %	Specificity %	PPV %	NPV %	Cohen's κ
ER (Allred ≥ 3 = positive)	22	22 (100.0)	100.0	100.0	100.0	100.0	1.00
PR (Allred ≥ 3 = positive)	22	22 (100.0)	100.0	100.0	100.0	100.0	1.00
HER2, exact 4-tier (0/1+/2+/3+)	22	17 (77.3)	N/A	N/A	N/A	N/A	0.67*
HER2, binary (0/1+ vs 2+/3+)	22	18 (81.8)	50.0	100.0	100.0	77.8	0.56

*Linear-weighted $\kappa = 0.80$; quadratic-weighted $\kappa = 0.90$ for the ordinal 4-tier HER2 comparison. N/A = not applicable to a multi-category comparison. Note: p63 concordance (6/6 paired cases, 100%) is reported in the results section rather than in this table, given its markedly smaller denominator.

Table 2. Details of the five HER2 discordant cases

Case (Sr. no.)	Frozen HER2	Biopsy HER2	FISH result	Section quality	Artefact (type)
1	1+	2+	Non-amplified	Good	Severe (freezing)
10	0	1+	Not done	Average	Moderate (freezing)
15	1+	2+	Non-amplified	Poor	Moderate (freezing)
19	1+	2+	Non-amplified	Average	Moderate (freezing)
26	1+	2+	Non-amplified	Poor	Severe (freezing)

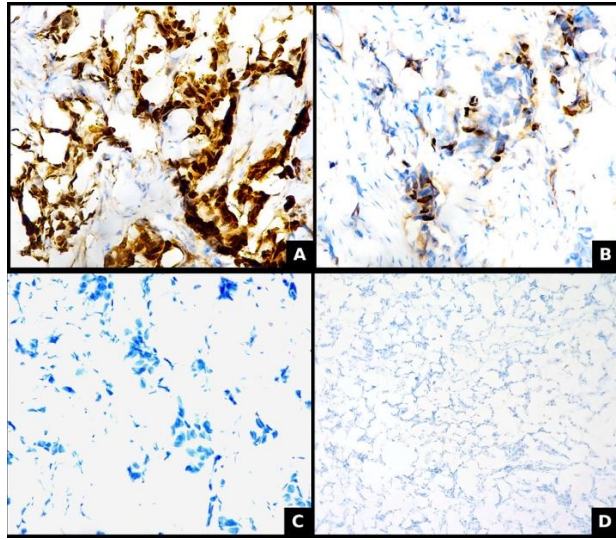


Figure 1. Representative photomicrograph of a case (immunohistochemistry, frozen section) showing (A) ER: positive (magnification x200); (B) PR: positive (magnification x200); (C) HER2: Negative (score 0) (magnification x200); (D) p63: negative, indicating the invasive phenotype of the tumor (magnification x100).

DISCUSSION

This prospective pilot study demonstrates that a frozen-section tissue-microarray platform can reproduce paraffin biopsy ER and PR results with complete concordance. It can also replicate HER2 category with substantial-to-almost-perfect agreement once the ordinal nature of HER2 scoring is taken into account, and provided equivocal (2+) results are confirmed by ISH as per standard practice. p63 behaved as a robust, reproducible confirmatory marker of the invasive phenotype on frozen tissue.

Nine of 35 cases (25.7%) could not be studied because the TMA cores underwent core fragmentation on further processing. Further, 4 of the remaining 26 cases (15.4%) were excluded from the paired concordance analysis: Two cases were exhausted during H&E sectioning and subsequent trimming, while the remaining two cores were non-representative. Freezing/ice-crystal artefact was the most common technical problem (66.7%), and poor quality correlated significantly with severe artefact ($p=0.03$), mirroring Zinzala et al.'s Indian audit, where frozen section detail was found slightly inferior to paraffin sections, with technical artefact accounting for the largest share (9.7%) of discrepancies¹³. Laucirica similarly identified

suboptimal freezing technique as a principal source of diagnostic error in intraoperative breast pathology⁵.

Our finding of full ER/PR concordance contrasts with the only closely comparable Western study, by Argon et al. from Turkey, who compared ER, PR, and HER2 staining between frozen and non-frozen sections of the same 53 tumors and found significantly reduced staining rate and intensity for all three markers after freezing, with some cases losing detectable staining altogether¹⁴. Methodological differences may explain our more favorable concordance: our comparator was a separately processed biopsy rather than a paired frozen/non-frozen section of the same block, our Allred cut-off of ≥ 3 is relatively insensitive to document small intensity shifts, and inter-laboratory antibody/retrieval differences are known to affect freezing-related antigen loss. Additionally, we learnt that a precise Allred score cannot be calculated as artefacts hinder the calculation of proportion score. Further, our positivity rates are also worth comparing against Indian and Western baselines, since a frozen-section assay is only as useful as its ability to reproduce a population's expected biology. Indian data have historically shown lower ER/PR positivity than Western series, Desai et al.'s pan-India series of 798 tumors found 32.6% ER and 46.1% PR positivity², and a South Indian series of 5,436 women showed a comparable pattern³, whereas Western cohorts typically report ER positivity above 70–80%. Our rates of 72.7% (ER) and 77.3% (PR) mimic the data from Western literature as well as contemporary Indian tertiary-care figures. These recent studies have reported comparably high hormone-receptor positivity (72.8% and 75%, respectively)^{15,16}, most plausibly reflecting case-mix rather than a frozen-section artefact, since biopsy positivity matched exactly.

Our exact four-tier HER2 concordance of 77.3% ($\kappa=0.67$) inclines toward the lower end of the range reported for TMA-based HER2 assessment. Furrer et al. reported 93.6% concordance ($\kappa=0.86$) comparing HER2 IHC on a diagnostic TMA core against a random additional block¹⁷, while a large Italian series found c-erbB2/HER2 concordance falling to 37% within the IHC 1+ category despite 97.8%/94.3% ER/PR concordance, mirroring our own pattern of perfect ER/PR agreement with more variable HER2 scoring¹⁸. HER2 discordance, in our study and elsewhere, clusters at the low-intensity end of the

- Frozen-section ER and PR immunohistochemistry showed complete concordance with paraffin biopsies, making it more reliable for determining receptor status rather than precise Allred scoring
- HER2 concordance was substantial, once its ordered scale was taken into account. However, a frozen-section score of 1+/2+ should not be read as reassuringly negative without ISH confirmation
- Thin TMA core was the main technical barrier, and therefore, careful core handling should be a priority in future studies

scale, where subtle membranous staining is most vulnerable to variation, whereas unambiguous 3+ or 0 results are highly reproducible. Because our binary sensitivity for the biopsy-positive/equivocal category was only 50.0%, therefore, using frozen section alone to decide eligibility for anti-HER2 neoadjuvant therapy is not advocated. A frozen-section score of 1+/2+ should not be treated as reassuringly negative and should still be confirmed against biopsy or ISH, as recommended by ASCO/CAP protocol¹¹.

p63 has been validated as a highly specific nuclear myoepithelial marker, superior to calponin and smooth-muscle myosin heavy chain for distinguishing invasive from in-situ or benign lesions, with only rare aberrant tumor-cell positivity in high-grade carcinomas¹². Its complete concordance and uniform negativity around invasive nests mirror the South Indian FFPE series¹⁹, but it rests on only 6 paired biopsy cases. It is important to note that p63 is not included in our breast panel and is requested only when there is a diagnostic dilemma between invasive malignancy and ductal carcinoma in situ, hence, it was performed in only 6 cases.

The principal limitations of this study are the single-centre design and the modest, paired sample (n=22). Further, the findings of this study are limited to upfront lumpectomies for invasive carcinoma of no special type (ductal), and their applicability to mastectomies, other histological subtypes, or low-cellularity tumors remains uncertain due to increased sampling error risks. Also, assembling a frozen-section tissue microarray adds significantly to time, cost, and labor alongside high core loss rates. Routine implementation seems to be difficult, and therefore, before putting into diagnostic use, a larger, adequately powered, multicentric study is needed to

validate the findings and understand the applicability of IHC on frozen section TMA in breast carcinoma.

CONCLUSIONS

In this pilot study, ER and PR immunohistochemistry on frozen-section tissue-microarray cores of treatment-naive breast carcinoma reproduced paraffin biopsy results with complete concordance. HER2 concordance was substantial to near-perfect once the ordinal nature of HER2 scoring was accounted for, with discordances confined to low-intensity categories that proved uniformly HER2-negative on reflex ISH. Freezing/ice-crystal artefact was the dominant technical limitation and correlated with poorer morphological quality. However, these findings are preliminary and justify a larger, adequately powered multicentric validation study before routine clinical adoption.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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Author Contributions

Conceptualization: NN, RR; Data collection: BS, NN, HG; Data analysis and/or interpretation: NN, HG, PS, AS; Writing - original draft preparation: BS, NN, RKP, SS; Writing - review and editing: NN, PS, RKP, SS; Figure preparation: BS, NN, AS; Project administration: NN, RR, RKP, SS.

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Declaration of Generative AI and AI-assisted technologies

This work was performed without the use of artificial intelligence (AI). No element of manuscript development involved AI.

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