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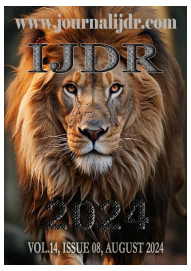

RESEARCH ARTICLE OPEN ACCESS

COMPARATIVE ANALYSIS OF EPITHELIAL-MESENCHYMAL TRANSFORMATION MARKERS IN PRIMARY FOCUS, TUMOR "BUDS" AND METASTATIC LYMPH NODES

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ABSTRACT

Background: Breast cancer is a heterogeneous disease with distinct molecular subtypes, each exhibiting unique clinical and pathological characteristics. The HER2-positive subtype, characterized by the overexpression of the HER2 protein, is associated with aggressive tumor behavior and poor prognosis. Epithelial-mesenchymal transition (EMT) is a critical process in tumor invasion, metastasis, and resistance to therapy, particularly in HER2-positive breast cancer. Tumor-infiltrating lymphocytes (TILs) play a crutial role in the host's immune response against cancer and have been correlated with clinical outcomes in various cancers, including breast cancer. ***Objective:*** This study aimed to investigate the phenotypic heterogeneity of EMT in different tumor sites, including the primary tumor focus, tumor "Buds," and metastatic lymph nodes in HER2-positive invasive ductal carcinoma (IDC). We focused on assessing the expression of EMT markers (E-cadherin, Beta-catenin, Vimentin) and their correlation with TILs, to understand their potential implications for clinical outcomes and personalized treatment strategies. ***Methods:*** A retrospective analysis was conducted on 130 cases of HER2-positive IDC. Archival formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 2018-2024 were selected based on strict inclusion criteria. Immunohistochemistry was performed to evaluate the expression of EMT markers and other relevant proteins, including Androgen Receptor, PD-L1, Ki67, and p53, across four tumor components: two primary tumor foci, tumor Buds, and metastatic lymph nodes. Quantitative data were analyzed using Spearman's rank correlation, Mann-Whitney U, and Kruskal-Wallis tests, with significance set at $p < 0.05$. *Results:* The study revealed significant heterogeneity in EMT marker expression across different tumor sites and stages. The mean percentage of TILs was highest at T1 stage (46.25%) and lowest at T4 stage (17.5%), with a strong negative correlation between TILs and tumor stage ($r = -0.599$). Vimentin expression showed a positive correlation with tumor stage, particularly in tumor Buds and lymph nodes, indicating a mesenchymal phenotype associated with advanced stages. E-cadherin expression decreased with tumor progression, with the lowest expression observed at T4 stage, suggesting a loss of epithelial characteristics. Beta-catenin expression increased across stages, with the highest levels in tumor Buds and lymph nodes at T4 stage. ***Conclusion:*** The findings suggest that EMT is a heterogeneous and stage-dependent process in HER2-positive IDC, with distinct expression patterns of EMT markers across different tumor sites. The correlations between EMT markers, TILs, and tumor stage underscore the potential of these markers as prognostic indicators and therapeutic targets. Identifying specific EMT profiles in different tumor microenvironments could enhance the development of personalized treatment strategies for patients with HER2-positive breast cancer, ultimately improving clinical outcomes. Further research is needed to elucidate the underlying mechanisms driving EMT heterogeneity and validate these findings in larger cohorts.

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INTRODUCTION

Breast cancer is a complex and heterogeneous disease with distinct molecular subtypes, each characterized by unique clinical, pathological, and molecular profiles (Richie, 200 and Andreopoulou, 2015). Among these subtypes, HER2-positive breast cancer stands out due to its aggressive nature and poor prognosis, often associated with resistance to conventional therapies (Abdou, 2022; Nami and Wang, 2017 & El Bairi, 2021). The HER2 (Human Epidermal Growth Factor Receptor 2) protein is overexpressed in approximately 15-20% of breast cancers, leading to uncontrolled cell growth and proliferation. Despite advancements in targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors, the prognosis for HER2-positive breast cancer patients remains challenging, particularly in cases involving invasive ductal carcinoma (IDC) (Slamon, 1979; Sapna, 2020; Nami, 2021). Epithelial-mesenchymal transition (EMT) is a critical biological process by which epithelial cells acquire mesenchymal traits, enhancing their migratory and invasive capabilities (Nami, 2021). EMT is implicated in various stages of cancer progression, including tumor invasion, metastasis, and resistance to therapy. In the context of HER2-positive breast cancer, EMT markers such as E-cadherin, Beta-catenin, and Vimentin have been associated with tumor aggressiveness, metastatic potential, and unfavorable clinical outcomes. Additionally, the tumor

microenvironment, including tumor-infiltrating lymphocytes (TILs), plays a crucial role in modulating tumor behavior and response to treatment (El Bairi, 2021). The present study aims to explore the phenotypic heterogeneity of EMT across different tumor sites, including the primary tumor focus, tumor "Buds," and metastatic lymph nodes in HER2-positive IDC. By employing a comprehensive immunohistochemical analysis, we evaluate the expression of EMT markers and their correlation with TILs, providing insights into the potential prognostic significance of these markers in different tumor microenvironments (Nami and Wang, 2017; Gámez-Chiachio et al., 2022; Venetis, 2022). Understanding the interplay between EMT markers, TILs, and tumor architecture could pave the way for more personalized therapeutic approaches, ultimately improving outcomes for patients with HER2-positive breast cancer (Slamon, 1989; Doroshow, 2021; Voorwerk, 2019; Aertgeerts, 2011).

MATERIALS AND METHODS

This retrospective study was based on archived material from the educational, scientific, and diagnostic laboratory of Tbilisi State Medical University. A retrospective study was performed to analyze the expression of epithelial-mesenchymal transition (EMT) markers and their association with tumor-infiltrating lymphocytes (TILs) in HER2-positive invasive ductal carcinoma (IDC.) The study was approved by the Ethics Commission of Tbilisi State Medical University. All methods were performed in accordance with the CAP guidelines and as approved by standard protocol for examination of resection specimens from patients with invasive carcinoma of breast (Version: 4.8.1).

Inclusion Criteria: Postoperative HER2-positive IDC patients were also evaluated for eligibility using immunohistochemical and histopathological diagnosis. Inclusion criteria:

HER2-positive molecular subtype invasive ductal carcinoma of the breast;

- Existence of the formalin-fixed and paraffin-embedded (FFPE) tissue blocks.

Cases of metastatic lymph node involvementLack of prior neoadjuvant chemotherapy to prevent therapy-related modifications in EMT marker levels.

A total of 130 patients were eligible. Patients had an average age of 42 years and all cases included in the study were node-positive.

Sample Collection and Processing: FFPE tissue blocks from the above mentioned cases were retrieved from the archival. A combination of two primary tumor foci, the advanced form as well as buds and metastatic nodes were chosen for evaluation. The absence of adjacent tumor formation on two primary foci, that were located at least 10 mm apart in order to facilitate independent sampling across different areas. Two tissue sections (4 µm thick) were cut from each FFPE block and mounted on glass slides for use in histological and immunohistochemical investigations.

Immunohistochemical Analysis: Immunohistochemistry was performed to assess the expression of EMT markers, including Vimentin, E-cadherin, and Beta-catenin, along with other relevant markers such as Androgen Receptor, PD-L1, Ki67, and p53. The following primary antibodies were used, all sourced from Leica Biosystems:

- **Vimentin (Clone V9)**: To assess mesenchymal differentiation.
- **E-cadherin (Clone NCH-38)**: To evaluate epithelial characteristics.
- **Beta-catenin (Clone 17C2)**: To investigate its role in cell adhesion and signaling.
- **Androgen Receptor (Clone AR441)**: To explore its expression in relation to EMT.
- **PD-L1 (Clone SP263)**: To assess immune checkpoint expression.
- **Ki67 (Clone MIB-1)**: As a marker of proliferation.
- **p53 (Clone DO-7)**: To evaluate the tumor suppressor status.

Antigen retrieval was performed using a microwave oven in citrate buffer (pH 6.0) for 20 minutes. The sections were then incubated with the primary antibodies at room temperature for 60 minutes, followed by the application of a polymer-based secondary antibody. Immunoreactivity was visualized using a DAB chromogen, and the slides were counterstained with hematoxylin.

Evaluation of Immunohistochemical Staining: The immunohistochemical staining was independently evaluated by two experienced pathologists blinded to the clinical data. The expression of EMT markers was quantified using a semi-quantitative scoring system that considered both the intensity of staining and the percentage of positively stained cells. The scoring was performed separately for the following four components:

- Primary Tumor Focus 1 (P1);
- Primary Tumor Focus 2 (P2);
- Tumor Buds (TB);
- Metastatic Lymph Nodes (LN);

The results were expressed as a mean score ± standard deviation for each tumor component. Discrepancies in scoring between the two pathologists were resolved by joint review and consensus.

Quantification of Tumor-Infiltrating Lymphocytes (TILs): TILs were quantified following the guidelines provided by the International TILs Working Group. The percentage of TILs was calculated by assessing the proportion of stromal area occupied by lymphocytes relative to the total stromal area. TIL evaluation was performed on the same tissue sections used for EMT marker analysis.

Statistical Analysis: Quantitative data were analyzed using IBM SPSS Statistics version 28.0.1.1. The following statistical tests were employed:

- **Spearman's rank correlation test**: To determine the correlation between EMT marker expression and clinical-pathological parameters, including tumor stage, TILs, and tumor "Buds."
- **Mann-Whitney U test**: To compare the distribution of EMT markers between different tumor sites.
- **Kruskal-Wallis test**: To evaluate the differences in EMT marker expression across various tumor stages.
- **Sensitivity and specificity analysis**: Calculated with 95% confidence intervals to assess the diagnostic value of EMT markers.

Only correlations and comparisons with a P-value <0.05 were considered statistically significant. All tests were two-tailed, and the results are presented as mean ± standard deviation unless otherwise stated.

RESULTS

We investigated the expression of EMT markers (Vimentin, E-cadherin, Beta-catenin) and its association with TILs in different tumor sites in 130 cases of HER2-positive invasive ductal carcinoma (IDC). The results have demonstrated substantial heterogeneity in the expression of EMT markers among different histological areas within a primary tumor focus, "Buds," as well as its matched metastatic lymph nodes which impact on local advancement and clinical consequences.

Tumor-Infiltrating Lymphocytes (TILs): The mean percentage of TILs across all stages of HER2-positive breast cancer was

30.8±13.39%. A stage-dependent decrease in TILs was observed, with the highest mean percentage at T1 (46.25%) and the lowest at T4 (17.5%). The negative correlation between TILs and tumor stage (r = -0.599) suggests that a higher TIL presence is associated with earlier stages of the disease.

Tumor "Buds": The average number of tumor "Buds" was 7.88 ± 4.63, with a positive correlation between the number of tumor Buds and vimentin expression in primary tumor focus 1 (r = 0.685) and lymph nodes (r = 0.747). This suggests that the formation of tumor "Buds" is linked to EMT, particularly the mesenchymal phenotype.

VimentinExpression: Vimentinexpression,indicative of mesenchymal characteristics, varied significantly between tumor sites. The highest mean expression was observed in tumor tissues (25 ± 16.55) and lymph nodes (22.8 ±14.43). A stage-dependent increase in vimentin expression was noted, with the highest levels at T4 (32.86 in tumor tissues). The strong positive correlation between vimentin expression and tumor stage (r = 0.599-0.601) underscores its role in tumor progression and metastasis.

expression and tumor stage (r = 0.816** in primary tumor) highlights its potential role in tumor progression and cellular migration, particularly in the context of tumor "Buds."

DISCUSSION

Our results reveal a marked phenotypic variation of EMT in HER2-positive breast cancer, evidenced by variable expression patterns of individual EMT markers among sites and stages from the same tumors. The high expression of vimentin showed a positive correlation with tumor stage, providing an evidence that EMT contributes to promoting the invasion and metastasis of tumors. On the other hand, decrease or loss E-cadherin expression during advanced state of tumor progression indicates that a gain from epithelial to mesenchymal (EM) transition is involved in tumorigenesis. The presence of tumor "Buds," concurrent with elevated vimentin and Beta-catenin, also suggest that these structures may act as intermediates in the metastatic cascade by serving to ease

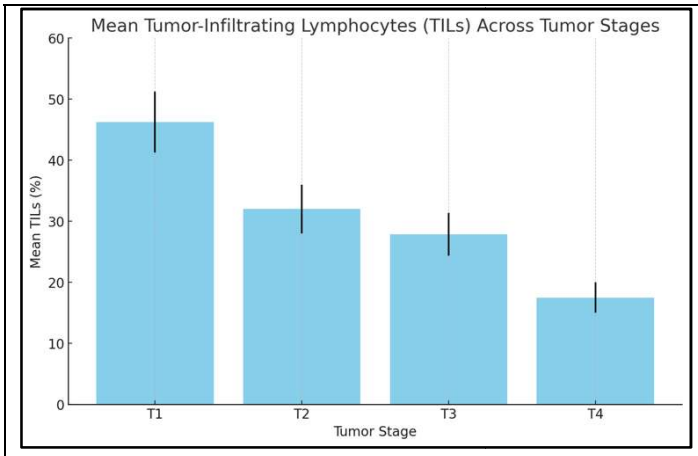

Figure 1. Displays the mean Tumor-Infiltrating Lymphocytes (TILs) across different tumor stages

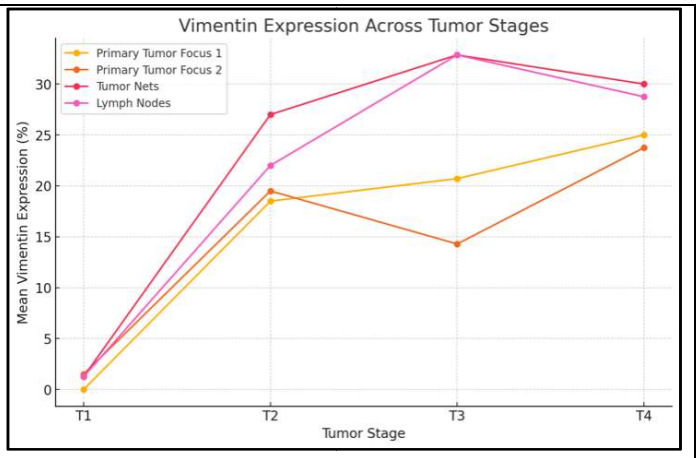

Figure 1. Line Plot (Vimentin Expression): Shows the Vimentin expression across different tumor components and stages.

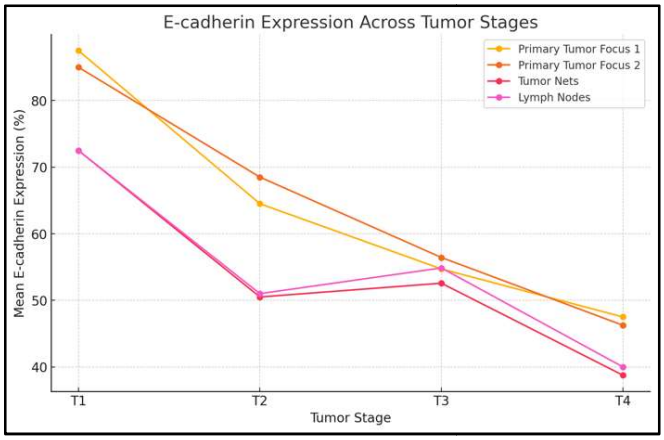

Figure 2. Line Plot (E-cadherin Expression): Illustrates the E-cadherin expression across different tumor components and stages

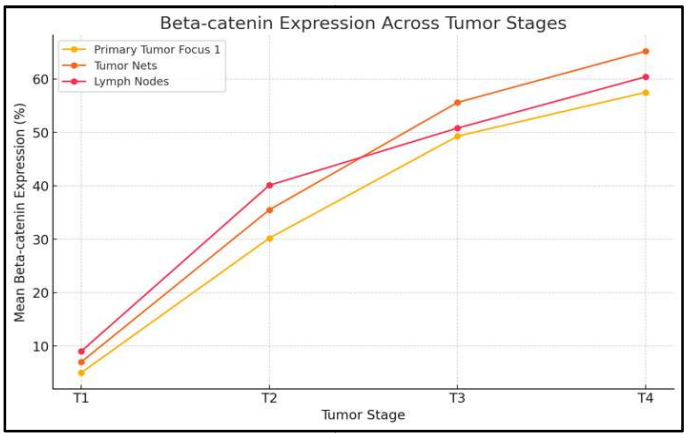

Figure 4. Line Plot (Beta-catenin Expression): Depicts the Beta-catenin expression across different tumor components and stages

E-cadherin Expression: E-cadherin, a marker of epithelial phenotype, showed an inverse relationship with tumor stage, with the highest expression at T1 (87.5 in primary tumor focus 1) and the lowest at T4 (47.5 in primary tumor focus 1). The negative correlation between E-cadherin expression and tumor stage (r = -0.571, p = 0.003) suggests that loss of epithelial characteristics is associated with advanced disease stages and may contribute to a more aggressive phenotype.

Beta-catenin Expression: Beta-catenin expression increased with tumor stage, with the highest mean expression at T4 (57.5 in primary tumor focus 1). The strong positive correlation between Beta-catenin

disseminating cancer cells away from the primary site (10). The inverse correlation between TILs and tumor stage indicated that a strong host immune response might be particularly important in the early stages of HER2-positive IDC to hinder tumorigenesis as well as metastasis. This may reflect the complexity of the tumor microenvironment in HER2-positive breast cancer that contributes to a heterogeneity in expression between sites. This heterogeneity can drive heterogeneous clinical outcomes in patients, and requires personalized therapies targeting individual EMT pathways.

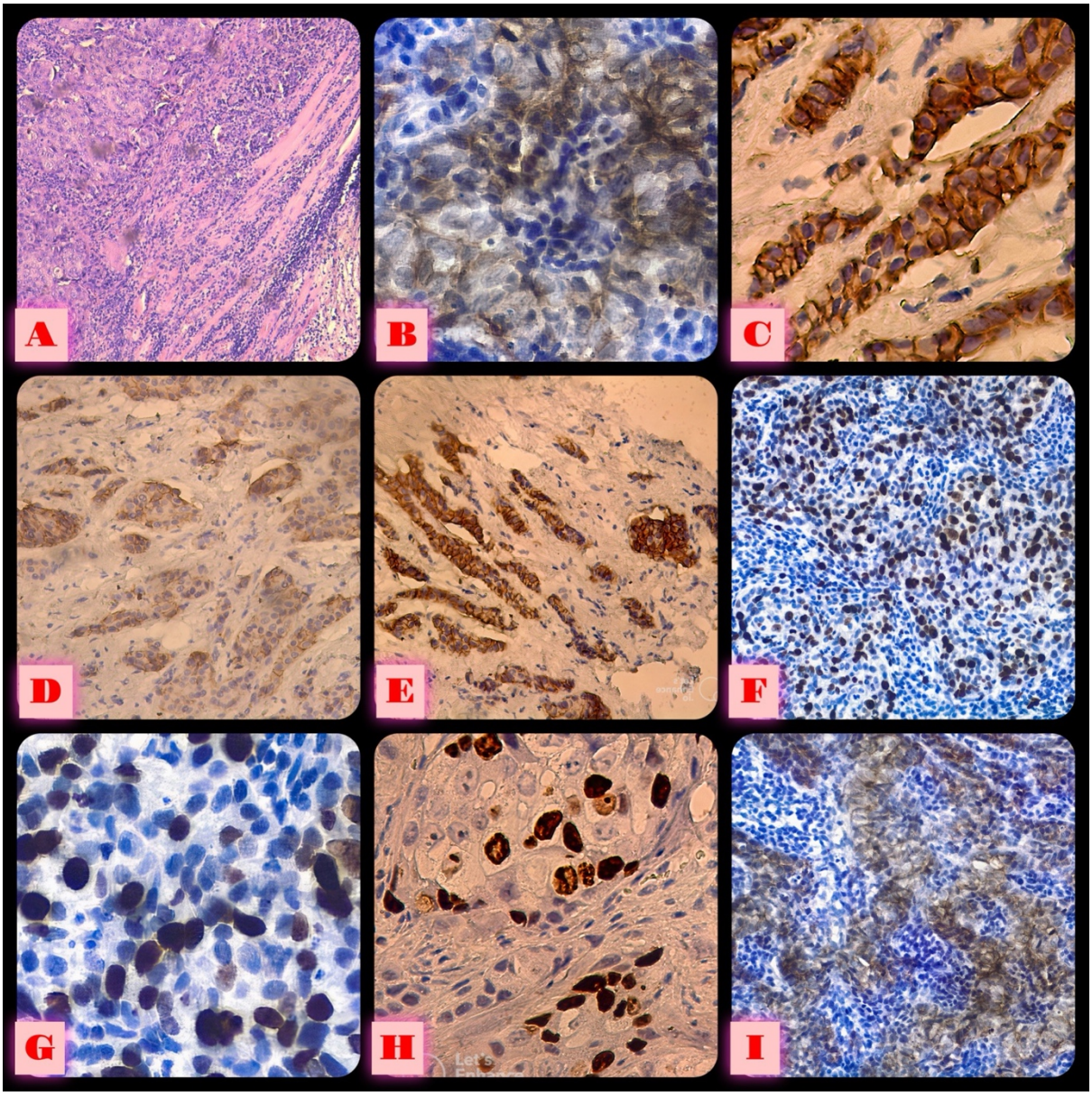

Figure 3. A. H&E technology: 100X. Tumor infiltrating lymphocytes 50%; B. IHC technology: 400X.Vimentin expression in tumor-grids'; C. IHC technology: 400X.E-cadherin expression in the main focus of the tumor; D. IHC technology: 200X. E-cadherin expression in tumor-grids F. IHC technology: 200X. beta-catenin expression in the main focus of the tumor; G. IHC technology: 400X. Beta-catenin expression in tumor tissues; H.IHC technology: 400X.KI67 expression in tumor tissues; I. IHC technology: 200X. Vimentin expression in the main focus of the tumor

CONCLUSION

This study shows that EMT is a dynamic and heterogeneous process in HER2-positive breast cancer, which varies considerably with marker expression across different anatomic sites and stages of disease. Taken together, the strong associations of EMT markers with tumour stage as well as TILs imply that these parameters are cooperative and conspire to drive aggressiveness of the tumor and modulate patient outcome. The present study indicates that EMT markers, especially vimentin, E-cadherin and Beta-Catenin may serve as potential prognostic strategies or therapeutic targets for HER2-positive breast cancer. Targeting therapy of EMT process and others developed therapies might be apply to the evidence-based, tumor site-tailored precision treatment that specific EMT profiles at different sites could demonstrate. Additional research is needed to really understand EMT heterogeneity and umph up the clinical relevance of these markers in wider patient cohorts. In conclusion, incorporation of EMT marker evaluation for individualized molecular therapy provides enhanced standards in the treatment strategies which yield a benefit to patients with HER2-positive breast cancer.

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