Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Histopathology-Based Molecular Subtyping and Immune Microenvironment Heterogeneity in Invasive Breast Carcinoma

Ahmed.j.mohammed
Email : ahmed.j.mohammed@uofallujah.edu.iq

Abstract

This study examined the relationship between immunohistochemistry-defined molecular subtypes of breast carcinoma and the tumour immune microenvironment. A total of 500 invasive breast carcinoma cases were retrospectively analysed using routine histopathology and immunohistochemistry. Tumours were classified into luminal A, luminal B, HER2-enriched, basal-like, and unclassified subtypes based on surrogate markers. Immune cell infiltration was assessed using CD8 and CD68 immunohisto-chemistry in a subset of cases, and immune-related gene expression was analysed by quantitative real-time PCR in selected CD8-dominant and CD68-dominant tumours. Basal-like tumours showed higher CD8 infiltration, while unclassified triple-negative tumours more frequently demonstrated macrophage-dominant immune profiles. Gene expression analysis showed higher expression of cytotoxic and T-cell–associated genes in CD8-dominant tumours compared with CD68-dominant tumours. These findings highlight immune heterogeneity across breast cancer subtypes and support the value of combining routine immunohistochemistry with targeted molecular analysis of invasive breast cancer

Key Words: Breast carcinoma; Histopathology; Immunohistochemistry; Molecular subtypes; Tumour microenvironment; Immune heterogeneity

الملخص

هدفت هذه الدراسة إلى تقييم العلاقة بين الأنماط الجزيئية لسرطان الثدي المحددة بواسطة الكيمياء المناعية النسيجية والبيئة المناعية للورم. تم تحليل ما مجموعه 500 حالة من سرطان الثدي الغازي بشكل استعادي باستخدام الفحص النسيجي الروتيني وتقنيات الكيمياء المناعية النسيجية. جرى تصنيف الأورام إلى الأنماط: لمعي**A** ، لمعي**B** ، غني بـ**HER2** ، شبيه القاعدي، وغير مصنّف، وذلك بالاعتماد على المؤشرات البديلة المعتمدة.

تم تقييم ارتشاح الخلايا المناعية باستخدام واسمَي **CD8** و **CD68**في مجموعة فرعية من الحالات، كما تم تحليل التعبير الجيني المرتبط بالمناعة باستخدام تفاعل البوليميراز المتسلسل الكمي في الزمن الحقيقي **(qRT-PCR)** في أورام مختارة ذات سيادة **CD8** أو **CD68.** أظهرت الأورام الشبيهة بالقاعدية مستويات أعلى من ارتشاح خلايا**CD8** ، في حين أظهرت الأورام الثلاثية السلبية غير المصنّفة نمطاً مناعياً يغلب عليه وجود الخلايا البلعمية.

كما أظهر تحليل التعبير الجيني زيادة في تعبير الجينات المرتبطة بالخلايا التائية والسمّية الخلوية في الأورام ذات السيادة **CD8** مقارنةً بالأورام ذات السيادة **CD68**. تسلط هذه النتائج الضوء على التغاير المناعي بين الأنماط المختلفة لسرطان الثدي، وتدعم أهمية دمج تقنيات الكيمياء المناعية النسيجية الروتينية مع التحليل الجزيئي الموجّه في دراسة سرطان الثدي الغازي

Introduction

Breast cancer is one of the most common malignancies affecting women worldwide and is characterised by marked biological and clinical heterogeneity. Tumours can be classified into distinct molecular subtypes based on the expression of hormone recep-tors and human epidermal growth factor receptor (HER2), including luminal A, luminal B, HER2-enriched, and triple-negative breast cancer (TNBC) [1]. This molecular heterogeneity has important implications for prognosis and therapeutic strategies, as subtypes differ in tumour biology, response to treatment, and survival outcomes [2]. The tumour microenvironment (TME) plays a critical role in breast cancer progression and treatment response, with immune cell composition influencing both disease course and therapeutic efficacy [3, 4]. Tumour-infiltrating lymphocytes (TILs), particularly CD8-positive cytotoxic T cells, have been

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

consistently associated with improved outcomes in several breast cancer subtypes, including TNBC and HER2-positive disease [4, 5]. High densities of CD8+ T cells have been linked to increased pathological complete response rates following neoadjuvant chemotherapy and better long-term survival in early TNBC patients [4]. In contrast, tumours with poor T cell infiltration are often described as "immune-cold" and tend to have less favourable prognostic features [3]. In addition to adaptive immune cells, innate immune cells such as tumour-associated macrophages (TAMs) are important components of the TME. Macrophages can exhibit diverse functions, including antitumour activity or promotion of tumour growth and immunosuppression, depending on their phenotype and localisation [6]. In TNBC, TAMs are often abundant and have been implicated in creating an immunosup-pressive microenvironment that can blunt effective antitumour immunity [7]. Despite substantial advances in breast cancer immunology, the interplay between molecular subtypes and immune infiltrates remains incompletely understood. Several studies have noted that immune infiltrates vary by subtype, with basal-like tumours generally showing higher overall immune cell presence and luminal tumours displaying lower infiltration levels [8, 9]. Immune profiling efforts, including multiplex immunohisto-chemistry and transcriptomic analyses, have begun to characterise the complex immune landscapes in different breast cancer subtypes, highlighting distinct patterns of T cell and macrophage infiltration [9-11]. Furthermore, integrating tumour immune pheno-types with molecular subtype classification has potential clinical relevance. Accurate classification of subtypes using immunohistochemistry (IHC) is widely used in routine practice, but the relationship between these surrogate markers and the underlying immune microenvironment has not been fully elucidated, particularly for TNBC subgroups [12]. This gap is especially important given the increasing interest in immu-notherapeutic strategies for breast cancer, where immune contexture may influence response to checkpoint inhibitors and other immunomodulatory agents [13-15]. Given this background, more comprehensive analyses that characterise both the surrogate molecular subtype and immune infiltration profiles are needed to better understand tumour biology and potentially inform therapeutic decision-making. In this study, we evaluated the immune microenvironment in an immunohistochemistry defined breast cancer cohort, focusing on CD8+ T cell and macrophage infiltration across subtypes and correlating these findings with gene expression related to immune activity.

Materials and Methods

Study design and case selection

This retrospective study included 500 newly diagnosed with invasive breast cancer, untreated invasive breast carcinomas collected over two years from a tertiary pathology centre. Cases with adequate tissue for histopathological,immunohistochemical, and molecular analyses were included, while recurrent, post-treatment, or poor-quality samples were excluded. Selected cases underwent additional immune profiling, and all analyses were performed on anonymised material in accordance with institutional ethical guidelines.

Specimen source and ethical approval

All tissue samples analysed in this study were obtained from St James's University Hospital, Leeds, through the diagnostic pathology services of the University of Leeds. The study was based on archived formalin-fixed, paraffin-embedded breast carcinoma specimens that had been collected as part of routine clinical diagnostic practice. Ethical approval for the

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

study was obtained from the relevant institutional research ethics committee before the work commenced (REC reference no. 20/YH/0185, approved in 2020). All samples were handled in accordance with established ethical guidelines for research involving human tissue. Patient identifiers were removed prior to analysis to protect confidentiality, and no direct patient contact or clinical intervention was involved in this study.

Histopathological evaluation and tumour grading

All cases were reviewed on routine haematoxylin and eosin–stained sections to confirm the diagnosis of invasive breast carcinoma. Tumours were classified according to standard histopathological criteria, and recognised special histological subtypes were recorded when present. Tumour grading was performed using a semi-quantitative approach based on the assessment of tubule formation, nuclear pleomorphism, and mitotic activity. Scores for each component were combined to assign an overall histo-logical grade. When multiple invasive tumour foci were identified, grading was based on the dominant invasive component. Other morphological features with known prognostic relevance were also assessed, including the presence of tumour necrosis, lymphovascular invasion, perineural invasion, and the degree of stromal lymphocytic response. Lymph node status was documented in cases where nodal tissue was available for evaluation.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using a polymer-based detection system routinely employed in diagnos-tic practice. Representative tumour blocks were selected, and 4-µm sections were cut, deparaffinised, rehydrated, and subjected to heat-induced antigen retrieval. Endogenous peroxidase activity was blocked before incubation with primary antibodies against ER, PR, HER2, Ki-67, EGFR, CK5/6, CD8, and CD68, using manufacturer-recommended protocols. Antibody binding was visualised with a horseradish peroxidase-labelled polymer system and diaminobenzidine, followed by haematoxylin counterstaining. Appropriate positive and negative controls were included in each run.

Digital slide scanning and immuno-histochemical scoring

All immunohistochemically stained slides were digitised using a high-resolution whole-slide scanner at ×40 magnification. Immunohistochemical assessment was performed using QuPath software, with tumour regions manually annotated to exclude non-neoplastic tissue and artefacts. Nuclear, membranous, and cytoplasmic markers were scored according to established diagnostic criteria, with thresholds applied consistently across cases. Digital scores were reviewed by pathologists, and final values were used for molecular subtype classification and analysis.

Definition of immunohistochemistry-based molecular subtypes

Breast carcinomas were classified into molecular subtypes using surrogate immuno-histochemical profiles based on hormone receptor status, HER2 expression, prolifera-tive index, and basal marker expression. Tumours were categorised as luminal A, luminal B, HER2-enriched, or triple-negative, with triple-negative cases further subdi-vided into basal-like or unclassified according to epidermal growth factor receptor and/or cytokeratin 5/6 expression. Subtype assignment was based on integrated digital immunohistochemical analysis and pathologist review.

Assessment of tumour-associated immune cell infiltration

Tumour-associated immune cell infiltration was evaluated using immunohistochemical staining

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

for CD8 and CD68 in order to assess cytotoxic T lymphocytes and macrophages within the tumour microenvironment. This analysis was performed on a subset of 50 cases, selected based on tissue availability and adequate staining quality. All assess-ments were carried out on digitised whole-slide images to allow consistent evaluation across cases. Representative tumour areas were selected for analysis, and regions showing necrosis, tissue artefact, in situ carcinoma, or non-neoplastic tissue were excluded. Immune cell infiltration was assessed across both intratumoural and stromal compartments in order to capture the heterogeneous distribution of immune cells within the tumour microenvironment. CD8-positive lymphocytes and CD68-positive macro-phages were identified based on their characteristic staining patterns and cellular morphology. Immune cell infiltration was assessed using a semi-quantitative approach that considered both the density and distribution of positive cells. Tumours were categorised as showing low, intermediate, or high levels of immune cell infiltration. Low infiltration was defined by sparse or occasional positive cells, intermediate infiltration by readily identifiable immune cells without dense clustering, and high infiltration by abundant immune cells with frequent clustering or diffuse stromal involvement. Initial scoring was performed on the digitised slides, and classification into low, intermediate, or high infiltration categories was independently carried out by two experienced pathologists who were blinded to the molecular subtype of the tu-mours. Any differences in scoring were resolved through joint review and consensus. The final immune infiltration categories were then used for subsequent analyses.

RNA extraction and quantitative Real-Time PCR analysis

RNA was extracted from FFPE tumour tissue using a commercial kit, with tumour-rich areas selected. Following quality assessment, quantitative real-time PCR was performed on 30 tumours (15 CD8-dominant and 15 CD68-dominant). cDNA was synthesised using standard protocols, and expression of IFNG, GZMB, CXCL9, and CXCL10 was analysed using GAPDH as the reference gene. Reactions were run in duplicate with appropriate controls, and relative expression was calculated using the ΔCt method.

Statistical analysis

Data were analysed using GraphPad Prism, with categorical variables sum-marised as frequencies and percentages and associations assessed using chi-square or Fisher's exact tests. All analyses were two-tailed, with $p < 0.05$ considered statistically signifi-cant, and missing data excluded without imputation.

Results

Clinical and Histopathological Characteristics

A total of 500 cases of invasive breast carcinoma were included in the analysis. Invasive carcinoma of no special type represented the majority of cases in the cohort. A smaller proportion consisted of recognised special histological subtypes, including invasive lobular carcinoma, mucinous carcinoma, tubular carcinoma, medullary carcinoma, metaplastic carcinoma, papillary carcinoma, and other less common tumour types. Evaluation of tumour differentiation showed that most cases were of intermediate histological grade. Well-differentiated and poorly differentiated tumours were less frequent. Although high-grade morphology was not the dominant pattern overall, it was consistently observed among histological subtypes known to be associated with more aggressive behaviour. Tumour necrosis was present in almost half of the cases, while lymphovascular invasion was identified in approximately one third of tumours. Perineu-ral

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

invasion was uncommon. Regional lymph node metastases were observed in more than half of the cases in which lymph nodes were available for assessment. The degree of stromal lymphocytic response varied across the cohort, ranging from minimal to prominent. A summary of histological subtypes, tumour grade, and associated patholog-ical features is presented in Table 1.

Table 1. Clinicopathological characteristics of the study cohort. The table summarises the distribution of histological subtypes, tumour grade, and associated pathological features among the analysed cases. Data are presented as number of cases and corresponding percentages

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Mixed lobular and ductal carcinoma	5	1.0
Mucinous carcinoma	6	1.2
Metaplastic carcinoma	3	0.6
Papillary carcinoma	3	0.6
Other rare subtypes*	4	0.8
Histological grade†		
Grade I	90	28.5
Grade II	181	57.3
Grade III	45	14.2
Tumour necrosis		
Present	231	46.2
Absent	269	53.8
Lymphovascular invasion		
Present	162	32.4
Absent	338	67.6
Perineural invasion		
Present	31	6.1
Absent	469	93.9
Lymph node status‡		
Positive	276	55.2
Negative	224	44.8

Hormone receptor and HER2 expression patterns

Immunohistochemical analysis showed variable expression of hormone receptors across the study cohort. Oestrogen receptor expression was identified in a substantial proportion of tumours and was characterised by nuclear staining within tumour cells. A representative example of ER nuclear positivity is shown in (Fig. 1A). ER expression was more commonly observed in well-differentiated and moderately differentiated tumours and was less frequent in high-grade carcinomas. Progesterone receptor expression showed a broadly similar distribution to ER, although discordant patterns were noted. PR positivity was observed in a subset of tumours that lacked ER expression, while some ER-positive tumours did not express PR. A representative example of

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

PR nuclear staining is shown in (Fig. 1B). Hormone receptor positivity was particularly frequent in tumours with lobular differentiation and other recognised special histological subtypes. HER2 immunoreactivity was identified in a distinct subset of cases and was characterised by strong, complete membranous staining of tumour cells. HER2 overexpression was more commonly seen in higher-grade carcinomas and in tumours with ductal morphology. A representative example of HER2 (3+) membranous staining is shown in (Fig. 1C). A proportion of tumours showed no expression of ER, PR, or HER2 and were classified as triple-negative. These tumours were more frequently associated with high-grade morphology and were analysed further for basal marker expression in subsequent sections.

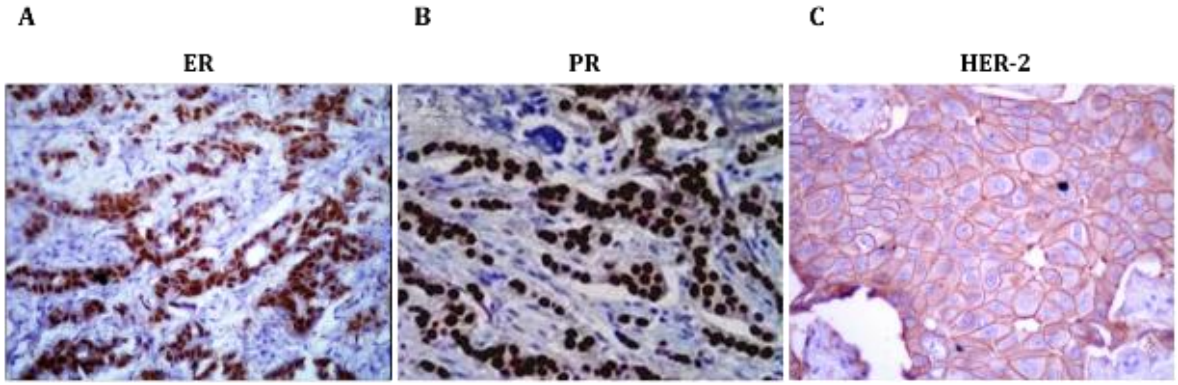

Figure 1: Representative immunohisto-chemical expression of hormone receptors and HER2 in invasive breast carcinoma. (A) Oestrogen receptor expression demonstrating nuclear immunoreactivity in tumour cells. (B) Progesterone receptor expression showing nuclear immunopositivity within neoplastic cells. (C) HER2 overexpression characterised by strong, complete circumferential membranous immunoreactivity in tumour cells. In all panels, positive immunohistochemical staining is visualised as brown chromogenic deposition, with nuclei counterstained blue using haematoxylin. Scale bars represent 50 μm, 40x.

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Proliferative activity and basal marker expression

Evaluation of tumour proliferative activity showed a wide range of Ki-67 expression across the cohort. Tumours within the hormone receptor–positive categories more often demonstrated lower proliferative indices, while increased Ki-67 labelling was mainly seen in higher-grade carcinomas. A representative example of high proliferative activity, with increased nuclear Ki-67 staining in tumour cells, is shown in (Fig. 2A). Triple-negative carcinomas were further assessed for the expression of basal lineage–associated markers. Epidermal growth factor receptor expression was identified in a subset of these tumours and was characterised by membranous staining of tumour cells (Fig. 2B). EGFR expression was not present in all triple-negative cases, indicating heterogeneity within this group. Cytokeratin 5/6 expression was also observed in a proportion of triple-negative tumours and was seen as cytoplasmic staining within tumour cells (Fig. 2C). Co-expression of EGFR and CK5/6 was identified in some cases, supporting classification as basal-like tumours. In contrast, other triple-negative carcinomas lacked expression of both basal markers and were categorised separately.

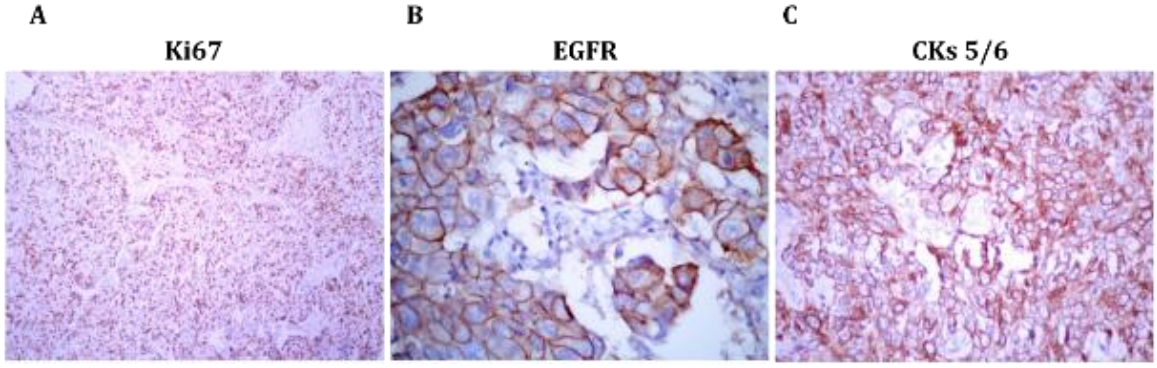

Figure 2: Representative immunohistochemical staining in invasive breast carcinoma showing Ki-67 proliferative activity (A) and basal marker expression by EGFR (B) and CK5/6 (C). Positive staining is brown with haematoxylin counterstain; scale bars, 50 µm, 40x.

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Distribution and Histopathological Associations of Molecular Subtypes

Classification of breast carcinomas using immunohistochemistry-based surrogate markers identified several distinct molecular subtypes within the study cohort. Tumours were grouped into luminal A, luminal B, HER2-enriched, basal-like, and unclassified categories based on combined assessment of hormone receptor status, HER2 expression, proliferative activity, and basal marker expression. Luminal A tumours represented the most common molecular subtype. These tumours were mainly associated with invasive carcinoma of no special type and invasive lobular carcinoma and were more often of low to intermediate histological grade. They also tended to show lower proliferative activity. Luminal B tumours formed a smaller proportion of cases and were characterised by higher proliferative indices, with a subset also demonstrating HER2 overexpression. HER2-enriched tumours were largely composed of invasive carcinomas of no special type and intraductal carcinomas and were more frequently associated with intermediate to high histological grade. Basal-like tumours were predominantly high-grade invasive carcinomas of no special type, with additional representation from medullary and metaplastic carcinomas. Tumours assigned to the unclassified subgroup showed a wide range of histological features, including both low-grade and high-grade morphologies. Statistical analysis showed significant associations between molecular subtype and several histopathological features, including histological subtype, tumour necrosis, stromal lymphocytic response, and lymphovascular invasion. No significant associations were found between molecular subtype and patient age, tumour size, or lymph node status. The distribution of histological subtypes across the molecular categories is summarised in Table 2

.

Table 2. Distribution of histological subtypes across immunohistochemistry defined molecular subtypes of breast carcinoma.

Histological subtype	L-A (%)	L-B (%)	HE2 (%)	TN (%)	Unclassified (%)
Invasive carcinoma (NST/IDC)	78.01	96.61	85.13	84.69	91.20
ILC	11.5	1.69	0.00	0.00	4.40

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Intraductal carcinoma	1.05	0.00	8.11	0.00	1.10
Tubular carcinoma	4.71	0.00	0.00	0.00	0.00
Papillary carcinoma	1.05	0.00	1.35	0.00	0.00
Mucinous carcinoma	2.09	1.69	1.35	0.00	0.00
Medullary carcinoma	0.00	0.00	2.71	8.24	1.10
Metaplastic carcinoma	0.00	0.00	0.00	3.53	0.00
Others	1.57	0.01	1.35	3.54	2.20

Immune cell infiltration patterns across immunohistochemistry-defined molecular subtypes

Assessment of tumour-associated immune cell infiltration using CD8 and CD68 immunohistochemistry, as evaluated by independent expert pathological review, revealed clear differences in immune patterns across immunohistochemistry-defined molecular subtypes of invasive breast carcinoma (Fig. 3). CD8-positive cytotoxic T-lymphocyte infiltration varied between molecular subgroups. Basal-like triple-negative breast carcinomas commonly showed intermediate to high levels of CD8 infiltration, with prominent accumulation of CD8-positive lymphocytes within the tumour stroma. In contrast, tumours within the unclassified triple-negative subgroup more often demonstrated low levels of CD8 infiltration. Luminal tumours, including luminal A and luminal B subtypes, were predominantly characterised by low CD8-positive immune cell presence, consistent with a relatively immune-poor tumour microenvironment. HER2-enriched tumours showed variable CD8 infiltration without a consistent dominant pattern (Fig. 3A). Evaluation of CD68-positive macrophage infiltration also demonstrated subtype-specific differences. Both basal-like and unclassified triple-negative breast carcinomas exhibited increased CD68-positive macrophage infiltration compared with luminal tumours. Unclassified triple-negative tumours more frequently showed macrophage dominant immune profile, whereas basal-like tumours displayed a more mixed immune composition. Luminal tumours were largely characterised by low CD68-positive macrophage infiltration, while HER2-enriched tumours tended to show

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

intermediate levels of macrophage presence (Fig. 3B). Focused analysis of the triple-negative cohort highlighted marked immune heterogeneity within this group. Basal-like tumours were characterised by relative enrichment of CD8-positive cytotoxic T lymphocytes, whereas unclassified triple-negative tumours more commonly demonstrated a macrophage-dominant tumour micro-environment, with increased CD68-positive infiltration and comparatively reduced CD8-positive lymphocytic presence (Fig. 3C–D). Together, these findings indicate that triple-negative breast carcinomas represent immunologically distinct entities beyond hormone receptor and HER2 status alone.

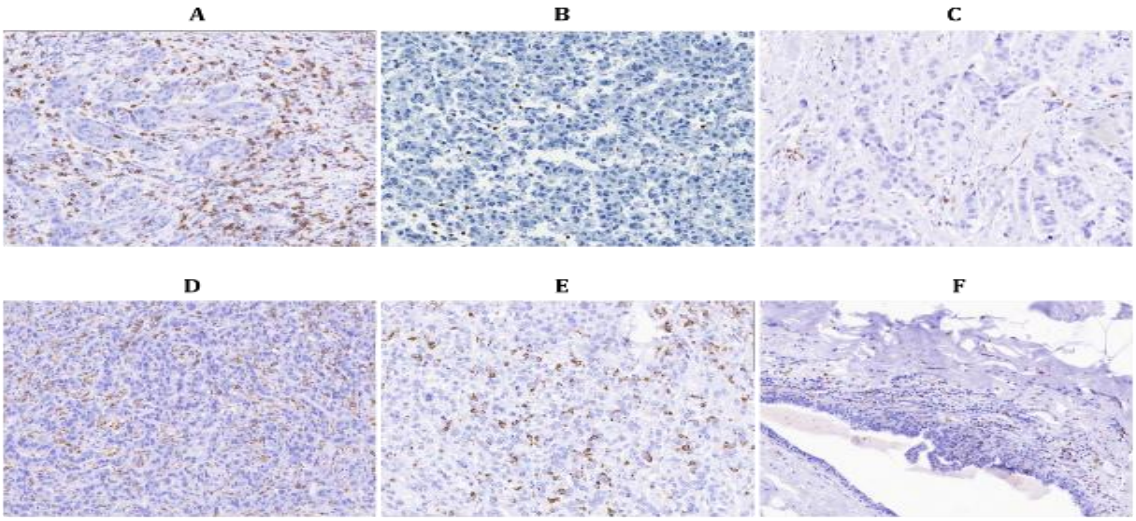

Figure 3: Representative CD8 and CD68 immunohistochemical staining demonstrating subtype-specific immune infiltration patterns in invasive breast carcinoma. Basal-like and unclassified triple-negative tumours show variable lymphocytic and macrophage infiltration, while luminal tumours display minimal immune cell presence. Positive staining is shown in brown with haematoxylin counterstain; images acquired at ×20 (field width 200 μm).

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Gene expression according to tumour immune phenotype

Quantitative real-time PCR was used to examine whether the immune phenotypes identified by CD8 and CD68 immunohistochemistry were reflected at the gene expression level. Based on immunohistochemical assessment, tumours were classified as CD8-dominant or CD68-dominant, and the expression of selected immune-related genes was analysed accordingly (Fig. 4). Tumours with a CD8-dominant immune phenotype showed lower ΔCt values for IFNG, indicating higher relative expression when compared with CD68-dominant tumours (Fig. 4A). A similar pattern was observed for GZMB, with increased expression in CD8-dominant tumours, consistent with greater cytotoxic immune activity (Fig. 4B). Analysis of chemokine expression showed comparable trends. CXCL9 expression was higher in CD8-dominant tumours than in CD68-dominant tumours, suggesting enhanced recruitment of T lymphocytes within this immune phenotype (Fig. 4C). Expression of CXCL10 followed the same pattern, with lower ΔCt values observed in CD8-dominant tumours relative to CD68-dominant tumours (Fig. 4D). Overall, these findings indicate that tumours characterised by a CD8-dominant immune infiltrate display a transcriptional profile consistent with increased cytotoxic immune activation and T-cell recruitment. In contrast, CD68-dominant tumours showed lower expression of these immune-related genes. These results support the immunohistochemical observations and further highlight functional immune heterogeneity within breast carcinoma

.

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

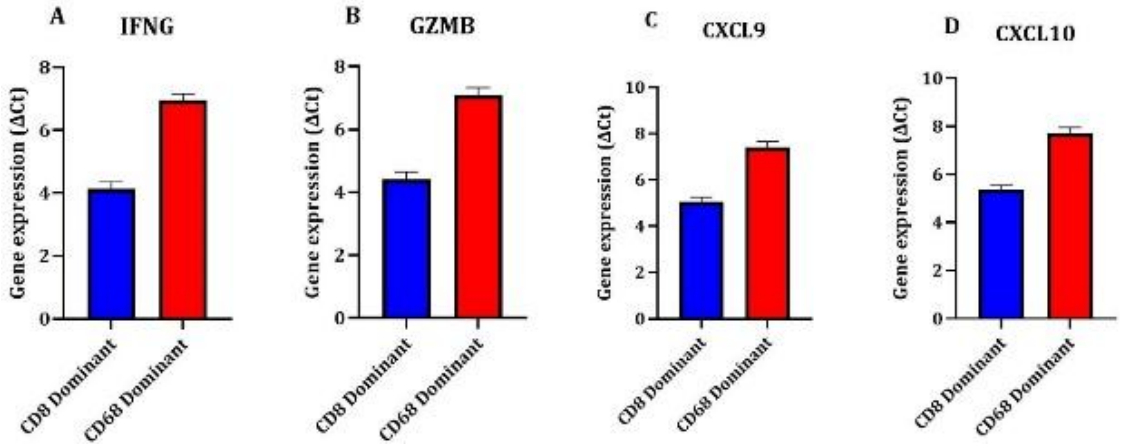

Figure 4. Immune-related gene expression according to tumour immune phenotype. Quantitative real-time PCR analysis of IFNG (A), GZMB (B), CXCL9 (C), and CXCL10 (D) in CD8-dominant and CD68-dominant tumours, expressed as ΔCt values normalised to GAPDH. Lower ΔCt values indicate higher expression. Bars represent mean values with variability between samples. CD8-dominant tumours show consistently lower ΔCt values, indicating enhanced cytotoxic immune activity.

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Discussion

In this study, we explored the relationship between immunohisto-chemistry defined molecular subtypes of breast cancer and the tumour immune microenvironment, with a particular focus on CD8-positive T lymphocytes and CD68-positive macrophages. By combining routine diagnostic immunohistochemistry with digital pathology and targeted gene expression analysis, we aimed to better understand immune heterogeneity across breast cancer subtypes, especially within triple-negative disease. Our findings confirm that immune infiltration is not evenly distributed across molecular subtypes. Basal-like triple-negative breast carcinomas showed higher levels of CD8-positive T-cell infiltration, whereas unclassified triple-negative tumours were more frequently characterised by prominent macrophage infiltration with relatively low CD8 presence. Luminal tumours, particularly luminal A cases, generally displayed low levels of both immune cell types. These observations are consistent with recent large-scale immune profiling studies showing that basal-like tumours tend to be more immune-active, while luminal tumours are often immune-poor [16-18]. An important aspect of our study is the separation of triple-negative breast cancer into basal-like and unclassified subgroups using surrogate immunohistochemical markers. Several recent studies have emphasised that TNBC is not a single entity but rather a collection of biologically distinct tumours with different immune landscapes [19, 20]. Our data support this view, showing that basal-like and unclassified TNBCs differ not only in marker expression but also in the composition of their immune microenvironment. The macrophage-dominant pattern observed in unclassified TNBC may reflect a more immunosuppressive tumour milieu, as suggested by experimental and clinical studies linking macrophage enrichment to immune evasion and tumour progression [21-23]. The gene expression analysis further supports the immunohistochemical findings. Tumours classified as CD8-dominant demonstrated higher expression of immune-related genes associated with cytotoxic activity and T-cell recruitment, including IFNG, GZMB, CXCL9, and CXCL10. In contrast, CD68-dominant tumours showed lower expression of these genes. Similar transcriptional patterns have been reported in recent transcriptomic studies comparing immune-hot and immune-cold breast tumours, reinforcing the idea that immune cell presence observed by immunohistochemistry reflects functional immune activity at the molecular level [24, 25]. From a practical perspective, our results highlight the value of routine immunohistochemistry in providing insight into the tumour immune context. While advanced genomic and single-cell technologies are increasingly used to study the tumour microenvironment, such approaches are not always available in routine diagnostic settings. The combination of standard markers such as CD8 and CD68 with digital image analysis offers a feasible way to gain meaningful

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

immune information from routinely processed tissue [26-28]. This approach may be particularly useful in settings where access to high-throughput molecular profiling is limited. There are, however, several limitations to this study. The immune and gene expression analyses were performed on subsets of the full cohort, which may limit the generalisability of the findings. In addition, the use of bulk RNA extracted from FFPE tissue does not allow precise attribution of gene expression to specific cell types. More detailed studies using spatial or single-cell approaches would be needed to fully dissect the functional interactions between tumour cells and immune populations [29-31]. Furthermore, clinical outcome data were not analysed in this study, and therefore no conclusions can be drawn regarding prognostic or predictive significance. Despite these limitations, our findings contribute to the growing body of evidence that breast cancer immune biology is closely linked to molecular subtype and that meaningful immune heterogeneity exists within triple-negative disease. Future studies integrating immune phenotyping with clinical outcomes and therapeutic response, particularly in the context of immunotherapy, may help refine patient stratification and guide treatment selection.

Conclusion

Breast carcinomas show subtype-specific immune heterogeneity, with differential CD8+ T-cell and CD68+ across immunohistochemistry defined groups, particularly within TNBC, highl-ighting the value of integrating routine histology, immunohistochemistry, and limited molecular analysis in everyday practice.

Authors' contributions

Ahmed J. Alfahdawi conceived and designed the study, performed the analyses, interpreted the data, and drafted and approved the final manuscript.

Acknowledgements

The authors thank the Section of Pathology, School of Medicine, University of Leeds, for technical support, and the Department of Pathological Analysis, College of Applied Sciences, University of Fallujah, for academic and infrastructural assistance.

References

1. Dieci, M.V., F. Miglietta, and V. Guarneri, *Immune Infiltrates in Breast Cancer: Recent Updates and Clinical Implications.* Cells,2021. **10**(2).doi: 10.3390/cells10020223
2. Tu, K.J., et al., *The tumor microenvironment of 14,837 breast cancers is associated with clinical outcome independently of genomic subtypes.* Cell Reports Medicine,2025. **6**(11): p. 102450.doi: https://doi.org/10.1016/j.xcrm.2025.102450
3. Moura, T., et al. *Breast Cancer and Tumor Microenvironment: The Crucial Role of Immune Cells*. Current Oncology, 2025. **32**, 143 doi: 10.3390/curroncol32030143
4. Rapoport, B.L., et al., *Tumor-Infiltrating Lymphocytes (TILs) in Early Breast*

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Cancer Patients: High CD3(+), CD8(+), and Immunoscore Are Associated with a Pathological Complete Response. Cancers (Basel),2022. **14**(10).doi: 10.3390/cancers14102525

5. Theodorou, S.D.P., et al. *Double–Multiplex Immunostainings for Immune Profiling of Invasive Breast Carcinoma: Emerging Novel Immune-Based Biomarkers*. International Journal of Molecular Sciences, 2025. **26**, 2838 doi: 10.3390/ijms26072838
6. Wu, T. and Y. Liao, *Roles of tumor-associated macrophages in triple-negative breast cancer progression.* Frontiers in Immunology,2025. **Volume 16 - 2025**.doi: 10.3389/fimmu.2025.1677363
7. Hao, D. and S. Chen, *Targeting tumor-associated macrophages in non-small cell lung cancer: mechanisms, prognosis, and therapeutic opportunities.* Frontiers in Immunology,2025. **Volume 16 - 2025**.doi: 10.3389/fimmu.2025.1679537
8. Torland, L.A., et al., *Multi-platform analysis of the tumor immune microenvironment associated with breast cancer subtypes.* Oncoimmunology,2026. **15**(1): p. 2610535.doi: 10.1080/2162402x.2025.2610535
9. Xu, T., et al., *Tumor-infiltrating immune cells state-implications for various breast cancer subtypes.* Frontiers in Immunology,2025. **Volume 16 - 2025**.doi: 10.3389/fimmu.2025.1550003
10. Chen, C.-H., et al., *Characterization of the tumor immune microenvironment in pregnancy-associated breast cancer through multiplex immunohistochemistry and transcriptome analyses.* Breast Cancer Research,2025. **27**(1): p. 154.doi: 10.1186/s13058-025-02097-4
11. Feng, D., et al., *CD8+ T-cell exhaustion: Impediment to triple-negative breast cancer (TNBC) immunotherapy.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer,2024. **1879**(6): p. 189193.doi: https://doi.org/10.1016/j.bbcan.2024.189193
12. Leeha, M., et al., *Immunohistochemistry-based molecular subtyping of triple-negative breast cancer and its prognostic significance.* Pathology and Oncology Research,2023. **Volume 29 - 2023**.doi: 10.3389/pore.2023.1611162
13. Yuan, Z., et al., *Research progress on immunotherapeutics for triple-negative breast cancer from a single-cell perspective.* Critical Reviews in Oncology/Hematology,2026. **218**: p. 105111.doi: https://doi.org/10.1016/j.critrevonc.2025.105111
14. Chen, F., et al., *Immune infiltration correlates with transcriptomic subtypes in primary estrogen receptor positive invasive lobular breast cancer.* npj Precision Oncology,2024. **8**(1): p. 257.doi: 10.1038/s41698-024-00746-z
15. Maynard, H., D. Liu, and J. Foldi, *Emerging Biomarkers to Predict Immunotherapy Response in Breast*

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

Cancer. Current Oncology Reports,2025. **27**(10): p. 1185-1207.doi: 10.1007/s11912-025-01710-1

16. Retecki, K., et al. *The Immune Landscape of Breast Cancer: Strategies for Overcoming Immunotherapy Resistance*. Cancers, 2021. **13**, 6012 doi: 10.3390/cancers13236012
17. Wang, T., et al., *Immunogenomic Landscape in Breast Cancer Reveals Immunotherapeutically Relevant Gene Signatures.* Front Immunol,2022. **13**: p. 805184.doi: 10.3389/fimmu.2022.805184
18. Wang, S., et al., *Clinical significance and immunogenomic landscape analyses of the immune cell signature based prognostic model for patients with breast cancer.* Brief Bioinform,2021. **22**(4).doi: 10.1093/bib/bbaa311
19. Koboldt, D.C., et al., *Comprehensive molecular portraits of human breast tumours.* Nature,2012. **490**(7418): p. 61-70.doi: 10.1038/nature11412
20. Herrera Juarez, M., et al., *Biology of the Triple-Negative Breast Cancer: Immunohistochemical, RNA, and DNA Features.* Breast Care (Basel),2020. **15**(3): p. 208-216.doi: 10.1159/000508758
21. Pollard, J.W., *Tumour-educated macrophages promote tumour progression and metastasis.* Nature Reviews Cancer,2004. **4**(1): p. 71-78.doi: 10.1038/nrc1256
22. Cassetta, L. and J.W. Pollard, *Targeting macrophages: therapeutic approaches in cancer.* Nature Reviews Drug Discovery,2018. **17**(12): p. 887-904.doi: 10.1038/nrd.2018.169
23. Lampiasi, N., *New Strategies for Macrophage Re-Education in Cancer: An Update.* International Journal of Molecular Sciences,2024. **25**(6): p. 3414.doi:
24. Rooney, M.S., et al., *Molecular and genetic properties of tumors associated with local immune cytolytic activity.* Cell,2015. **160**(1-2): p. 48-61.doi: 10.1016/j.cell.2014.12.033
25. Thorsson, V., et al., *The Immune Landscape of Cancer.* Immunity,2018. **48**(4): p. 812-830.e14.doi: 10.1016/j.immuni.2018.03.023
26. Mei, S., et al. *An Immune Gene Signature Stratifies Breast Cancer Prognosis Through iCAF-Driven Immunosuppressive Microenvironment*. Biomedicines, 2025. **13**, 2966 doi: 10.3390/biomedicines13122966
27. Zhang, X.M., et al., *Artificial intelligence in digital pathology diagnosis and analysis: technologies, challenges, and future prospects.* Mil Med Res,2026. **12**(1): p. 93.doi: 10.1186/s40779-025-00680-6
28. Piccione, J., et al., *Digital pathology in veterinary clinical pathology: A review.* Vet Pathol,2025. **62**(5): p. 631-645.doi: 10.1177/03009858251334340
29. Kilgallon, A., R. Tubelleza, and A. Kulasinghe, *Large-scale spatial profiling of the tumor microenvironment.* Cell,2026. **189**(2): p. 343-344.doi: https://doi.org/10.1016/j.cell.2025.12.028

Al-Shatrah Veterinary and Biological Sciences Journal
ISSN (Print): 2958-8952
Volume 2, Issue 2
Page 20 - 37

30. Wu, S.Z., et al., *A single-cell and spatially resolved atlas of human breast cancers.* Nature Genetics,2021. **53**(9): p. 1334-1347.doi: 10.1038/s41588-021-00911-1

31. Xu, Y., et al., *Current Status of Breast Cancer Immunotherapy and Prognosis-Related Markers.* Breast Cancer (Dove Med Press),2025. **17**: p. 339-348.doi: 10.2147/bctt.S506949