A key genomic subtype associated with lymphovascular invasion in invasive breast cancer

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BACKGROUND: Lymphovascular invasion (LVI) is associated with the development of metastasis in invasive breast cancer (BC). However, the complex molecular mechanisms of LVI, which overlap with other oncogenic pathways, remain unclear. This study, using available large transcriptomic datasets, aims to identify genes associated with LVI in early-stage BC patients.

METHODS: Gene expression data from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort (n = 1565) was used as a discovery dataset, and The Cancer Genome Atlas (TCGA; n = 854) cohort was used as a validation dataset. Key genes were identified on the basis of differential mRNA expression with respect to LVI status as characterised by histological review. The relationships among LVI-associated genomic subtype, clinicopathological features and patient outcomes were explored.

RESULTS: A 99-gene set was identified that demonstrated significantly different expression between LVI-positive and LVI-negative cases. Clustering analysis with this gene set further divided cases into two molecular subtypes (subtypes 1 and 2), which were significantly associated with pathology-determined LVI status in both cohorts. The 10-year overall survival of subtype 2 was significantly worse than that of subtype 1.

CONCLUSION: This study demonstrates that LVI in BC is associated with a specific transcriptomic profile with potential prognostic value.

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BACKGROUND
Outcomes for early-stage breast cancer (BC) patients have improved over recent decades as a result of better diagnostic accuracy, targeted drug therapies, in addition to improvements in early diagnosis.1 However, the ten-year mortality rates of BC patients remain ~20% which is attributable to the development of metastasis.2 Several histopathological features have been studied as prognostic factors in BC, including tumour size, lymph node status and histological grade,3–5 which are strongly associated with outcome. Lymphovascular invasion (LVI) is an early event in the development of metastasis and is a potent prognostic factor.6 Although the molecular profiles associated with tumour differentiation in terms of histological type and grade and development of lymph node metastasis have been well characterised,7–9 the molecular mechanisms of LVI and associated genes that may represent therapeutic targets or biomarkers remain to be identified. The main challenge in determining the molecular profiles associated with LVI status in BC stems from the lack of LVI status in the available large-scale molecular studies in addition to the inherent subjectivity of morphological assessment of LVI status.

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC)10 and The Cancer Genome Atlas (TCGA)11 cohorts are currently the largest genomic and transcriptomic datasets of early-stage BC patients with clinical follow-up. In this study, using these large transcriptomic datasets combined with thorough histological assessment of LVI, we applied bioinformatic analysis to evaluate the genes associated with LVI and assessed the prognostic value of genomic subtype based on LVI status.

METHODS
The METABRIC cohort
In the METABRIC study,10 mRNA was extracted from primary tumours of female patients, and mRNA expression was evaluated using the Illumina TotalPrep RNA Amplification Kit and Illumina Human HT-12 v3 Expression BeadChips (Ambion, It is also possible that the text may contain some errors or inconsistencies, such as incorrect dates or references. It is important to carefully review the original source for the most accurate information.
RESULTS
Clinopathological and prognostic significance of LVI status
In the METABRIC cohort, 635/1,565 (41%) were LVI-positive and 930 (59%) were LVI-negative. The LVI-positivity rate was 41.1% (117/285) in the Nottingham cases and 40.5% (518/1,280) in the remaining METABRIC cases. In the TCGA cohort, 295/854 (35%) patients were LVI-positive and 559 (65%) were LVI-negative. In both cohorts, LVI positivity was significantly associated with large tumour size (METABRIC: \( p < 0.0001 \); TCGA: \( p = 0.00055 \)), positive nodal status (METABRIC and TCGA: both \( p < 0.0001 \)) and high histological grade (METABRIC and TCGA: both \( p < 0.0001 \); Supplementary Table 3).

The survival of LVI-positive BC patients was significantly worse compared with LVI-negative patients in the METABRIC (hazard ratio (HR) 1.70, 95% CI 1.45–2.01, \( p < 0.0001 \); Fig. 1a) and TCGA cohorts (HR 2.2, 95% CI 1.46–3.38, \( p = 0.0019 \); Fig. 1b). Univariate and multivariate analyses of both METABRIC and TCGA datasets are summarised in Supplementary Table 4. Univariate analysis using the Cox proportional hazards regression model identified LVI-positive status, large tumour size (METABRIC: HR 1.82, 95% CI 1.49–2.21, \( p < 0.0001 \); TCGA: HR 1.81, 95% CI 1.08–3.04, \( p = 0.025 \)), positive nodal status (METABRIC: HR 2.06, 95% CI 1.74–2.44, \( p < 0.0001 \); TCGA: HR 1.85, 95% CI 1.20–2.85, \( p = 0.0056 \)), negative ER status (METABRIC: HR 1.66, 95% CI 1.38–1.99, \( p < 0.0001 \); TCGA: HR 1.89, 95% CI 1.19–2.98, \( p = 0.0065 \)) and negative PR status (METABRIC: HR 1.67, 95% CI 1.42–1.98, \( p < 0.0001 \); TCGA: HR 1.68, 95% CI 1.08–2.61, \( p = 0.020 \)) as poor prognostic factors in both cohorts. In addition, significant prognostic factors included high histological grade (HR 1.63, 95% CI 1.37–1.93, \( p < 0.0001 \)) and positive HER2 status (HR 1.92, 95% CI 1.54–2.38, \( p < 0.0001 \)) in the METABRIC cohort. LVI positivity was an independent poor prognostic factor in multivariate analysis (METABRIC: HR 1.29, 95% CI 1.07–1.56, \( p = 0.0073 \); TCGA: HR 2.19, 95% CI 1.32–3.62, \( p = 0.0023 \); Supplementary Table 4).

Genes associated with LVI
The overlapping DEGs between (1) the Nottingham cases in the METABRIC cohort (\( n = 285 \)) and (2) other METABRIC cases (\( n = 1280 \)) are shown in Supplementary Tables 1 and 2. Overlapping DEGs between the two groups were included in the gene set associated with LVI.

The Cluster 3.0 package was used for clustering and heat map construction. Clustering analysis was performed using METABRIC as the discovery set and validated using TCGA data as the validation set. TCGA mRNA data were log2-transformed prior to clustering analysis.

For pathway analysis, the WEB-based GeNE SeT AnaLysis Toolkit (WebGestalt) was used to calculate significantly enriched gene ontologies and pathways associated with these genes. The false discovery rate was controlled using the Benjamini–Hochberg procedure in WebGestalt, with an adjusted-\( p < 0.01 \) considered statistically significant.

Statistical analysis
Statistical analyses were conducted using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA). The chi-squared test was used to assess differences among several clinicopathological factors, including LVI status, tumour size, lymph node status, histological grade, oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2) and molecular subtypes, as stratified by the LVI-associated genomic subtype.

Kaplan–Meier survival curves of 10-year overall survival (OS) were plotted for the METABRIC and TCGA cohorts. The 10-year OS in this study was defined as the day of death within 10 years or the day of completing follow-up from the day of surgery. In univariate and multivariate analyses, 95% confidence intervals (CIs) were assessed using the Cox proportional hazards regression model to determine the associations between clinicopathological factors (LVI status, tumour size, lymph node status, histological grade, ER, PR and HER2), including the LVI-associated genomic subtype and prognosis.

In the METABRIC and TCGA cohorts, subtype 2 was significantly associated with large tumour size (both \( p < 0.0001 \)), high histological grade (both \( p < 0.0001 \)), ER negativity (both \( p < 0.0001 \)), PR negativity (both \( p < 0.0001 \)) and HER2 positivity (both \( p < 0.0001 \)}.
Interestingly, 69% of luminal B, 95% HER2-enriched and 90% basal-like BC were classified as subtype 2 in the METABRIC cohort. Patients with LVI-related subtype 2 had a significantly worse prognosis compared with those presenting with subtype 1 tumours in both cohorts (METABRIC: HR 1.78, 95% CI 1.50–2.12, \( p < 0.0001 \); TCGA: HR 2.32, 95% CI 1.35–3.99, \( p = 0.0023 \); Fig. 1c, d). In multivariate survival analysis, the LVI-related genomic subtype was an independent poor prognostic factor in both cohorts (METABRIC: HR 1.32, 95% CI 1.07–1.63, \( p = 0.0098 \); TCGA: HR 2.76, 95% CI 1.19–6.38, \( p = 0.018 \); Fig. 3 and Supplementary Table 7).

**DISCUSSION**

In this study, we identified a 99-gene set significantly associated with LVI status in the METABRIC dataset. We validated this finding using the TCGA dataset. LVI is a biomarker for aggressive BC and is considered predictive for metastasis. Patients with LVI-related subtype 2 had a significantly worse prognosis compared with those presenting with subtype 1 tumours in both cohorts (METABRIC: HR 1.78, 95% CI 1.50–2.12, \( p < 0.0001 \); TCGA: HR 2.32, 95% CI 1.35–3.99, \( p = 0.0023 \); Fig. 1c, d). In multivariate survival analysis, the LVI-related genomic subtype was an independent poor prognostic factor in both cohorts (METABRIC: HR 1.32, 95% CI 1.07–1.63, \( p = 0.0098 \); TCGA: HR 2.76, 95% CI 1.19–6.38, \( p = 0.018 \); Fig. 3 and Supplementary Table 7).

The underlying molecular mechanisms driving LVI in BC, which are potential therapeutic targets, have yet to be identified. The 99 genes in the LVI-related gene signature from this study are significantly associated with extracellular pathways. In previous work, Klahan et al. suggested their gene set associated with LVI was related to extracellular matrix components using microarray data from 108 BC patients. Epithelial–mesenchymal transition (EMT)-implicated genes in prostate cancer have also been associated with pathways relating to the extracellular space.
The extracellular matrix comprises a network of structural proteins, and reorganisation of this matrix is required for cancer to progress. The EMT is thought to play an important role in the process of metastasis to distant sites, and certain EMT markers are related to LVI status in BC. In the 99 gene LVI signature set, there are several genes associated with extracellular pathways that are implicated in BC prognosis. For example, heat shock protein (HSPB1), is associated with BC aggressiveness and metastasis. HSPB1 expression is upregulated in the early phase of cell differentiation, which implies that HSPB1 may play an important role in controlling the growth and migration of cancer stem-like cells. Another example is apolipoprotein C1 (APOC1), which is considered as a prognostic biomarker for triple-negative BC. APOC1 is thought to regulate the inflammatory response in cancer tissues, which may be closely related to the elimination of proliferating cancer cells. Upregulation of MMPs is also related to cancer cell proliferation, invasion and epithelial-to-mesenchymal transformation and is indicative of a poor prognosis for BC patients. As an example, MMP-11, which belongs to the MMP family, promotes BC development by inhibiting apoptosis as well as enhancing the migration and invasion of BC cells.

Comparison of the METABRIC and TCGA cohorts was a limiting factor in this study, in terms of the different methods used to quantify and statistically analyse gene expression and in the approaches to LVI evaluation. We previously developed a method for the accurate detection of LVI using immunostaining for CD34 or D2-40. In the Nottingham cases, we evaluated LVI status using strict criteria based on both morphology and immunohistochemistry. However, for the TCGA BRCA cohort, we evaluated LVI status using H&E-stained slides alone from the cBioPortal database. Although LVI evaluation using only one H&E slide is feasible, it may be difficult to clearly identify LVI negativity. In present study, the LVI-positivity rates were

Table 1. List of 99 genes significantly associated with lymphovascular invasion

| Genes | Genes | Genes |
|-------|-------|-------|
| Upregulated genes | Downregulated genes | Upregulated genes |
| APOC1  | KRT7  | UCP2  |
| APOE   | KRT8  | YWHAZ |
| CALM5  | LAPTM2B | ANXA1 |
| CCNB2  | LRR2C6 | CS  |
| CDCA5  | LY6E  | CDC42EP4 |
| COX6C  | MPP11  | CEBPβ |
| DNAJA4 | MK1   | CFβ  |
| EEF1A2 | NME1  | CFD  |
| ELF3   | NOP56  | CLC6 |
| ERBB2  | PGAP3  | CXCL12 |
| GNA5   | PITX1  | CXCL14 |
| HMGA1  | PTTG1  | CYBDR1 |
| HMG13  | S100P  | CYPA4X1 |
| HSPB1  | SCD   | DCN   |
| IDH2   | SLC52A2 | DCRK |
| IFI17  | SLC9A3R | DPI3L2 |
| ISG15  | SPDEF  | DUSP1 |
| KRT18  | TM7SF2 | EEF1B2 |
| KRT18P55 | UBE2C  | FBN1 |
| KRT19  | UBE2S  | FCER1A |
|        |       |       |

Table 2. Gene ontology pathways significantly associated with 99 genes related to lymphovascular invasion

| Ontology Name | Genes in Ontology | Ontology Space | Genes | p-value | Genes | Genes | Genes | Genes |
|---------------|-------------------|----------------|-------|---------|-------|-------|-------|-------|
| Extracellular space | 1350 | GO0005615 | 23 | 6.52 | KRT7, KRT8, CD34, FDLN1, D2-40, S100A4, S100P, S100A3 |
| Blood microvascular | 110 | GO0045562 | 7 | 0.52 | CEBPβ, CDCA5, ANG, MMP11, CDC42EP4, MKP1, EEF1A2 |
| Extracellular matrix | 500 | GO0000112 | 11 | 2.37 | MPP11, S100A4, S100P, ADAM10, MMP11, CDC42EP4, MKP1, EEF1A2 |
closely similar between the Nottingham cases, the remaining METABRIC cases and TCGA_BRCA cases using the different LVI-evaluations. Although our results might suggest the adequacy of LVI evaluation with only one H&E-stained slide, further analysis with the larger cohorts to assess the LVI status using both H&E and IHC slides is necessary to report accurately on LVI status.

Microarrays were used to evaluate mRNA expression in the METABRIC analysis. In contrast, RNA-seq using NGS was used in the TCGA analysis. Microarray platforms have been used and validated for nearly two decades, and this approach has been widely used for evaluating multi-gene expression. Conversely, the unbiased genome-wide RNA-seq method allows for the analysis of all annotated transcripts in addition to the identification of novel transcripts, splice junctions and noncoding RNAs. These technological and methodological differences may underpin the known challenges of relating microarray and RNA sequencing data between studies.\textsuperscript{35,36} For example, the different approaches can have different lower limits of detection or may encompass different genomic regions. Thus, we cannot assume that the methods are interchangeable, and doing so would require rigorous cross-assay comparisons.\textsuperscript{\textsuperscript{37}} Although there is statistical agreement across the different cohorts in the present study, further analysis using identical technologies (microarray and/or NGS assays) may provide clearer validation of the LVI gene signature.

In conclusion, we have confirmed the suitability and prognostic significance of our LVI-evaluation approach using the METABRIC and TCGA cohorts. We have determined genomic subtype associated with LVI status and patient outcome in BC, therefore, providing an experimental tool which may serve to unravel the complex gene networks associated with LVI with potential clinical relevance. Consistency between clinical cohorts stratified by LVI-gene signature may be further

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Fig. 2 Cluster analysis of the gene set associated with LVI. a The dendrogram of 99 LVI-related genes using METABRIC cohort, in which the pattern of the branches indicates the relationship for each gene. Heat maps in accordance with the LVI-related gene set for the b METEBRIC and c TCGA cohorts showed that all cases were clearly divided between subtypes 1 and 2 using cluster analysis.
| Factors                  | METABRIC cohort LVI-associated genomic subtypes | p-value | TCGA cohort LVI-associated genomic subtypes | p-value |
|-------------------------|-----------------------------------------------|---------|--------------------------------------------|---------|
|                         | Subtype 1 | Subtype 2 | Total          | Subtype 1 | Subtype 2 | Total          |
| LVI                     |           |           |                |           |           |                |
| Positive                | 262 (35.5%) | 373 (45.1%) | 635 <0.0001   | LVI       | Positive   | 61 (23.2%) | 234 (39.6%) | 295 <0.0001 |
| Negative                | 476 (64.5%) | 454 (54.9%) | 930           | Negative  | 202 (76.8%) | 357 (60.4%) | 559           |
| Tumour size             |           |           |                |           |           |                |
| ≥2 cm                   | 454 (61.9%) | 613 (75.2%) | 1067 <0.0001  | Tumour size | T 2–4 | 164 (62.4%) | 451 (76.3%) | 615 <0.0001 |
| <2 cm                   | 279 (38.1%) | 202 (24.8%) | 481           | T 1       | 99 (37.6%)  | 140 (23.7%) | 239           |
| Nodal status            |           |           |                |           |           |                |
| Positive                | 307 (41.7%) | 428 (51.9%) | 735 <0.0001   | Nodal status | Positive | 128 (48.9%) | 295 (50.3%) | 423 0.71 |
| Negative                | 429 (58.3%) | 396 (48.1%) | 825           | Negative  | 134 (51.1%) | 292 (49.7%) | 426           |
| Histological grade      |           |           |                |           |           |                |
| Grade 3                 | 187 (26.5%) | 586 (72.8%) | 773 <0.0001   | Histological grade | Grade 3 | 28 (11.3%)  | 324 (56.9%) | 352 <0.0001 |
| Grade 1, 2              | 519 (73.5%) | 219 (27.2%) | 738           | Grade 1, 2 | Grade 1, 2 | 219 (88.7%) | 245 (43.1%) | 464           |
| ER                      |           |           |                |           |           |                |
| Positive                | 707 (95.8%) | 497 (60.1%) | 1204 <0.0001  | ER       | Positive   | 246 (97.6%) | 393 (68.7%) | 185 <0.0001 |
| Negative                | 31 (4.2%)  | 330 (39.9%) | 361           | Negative  | 6 (2.4%)   | 179 (31.3%) | 639           |
| PR                      |           |           |                |           |           |                |
| Positive                | 533 (72.2%) | 295 (35.7%) | 828 <0.0001   | PR       | Positive   | 235 (94.0%) | 311 (54.8%) | 546 <0.0001 |
| Negative                | 205 (27.8%) | 532 (64.3%) | 737           | Negative  | 15 (6.0%)  | 257 (45.2%) | 272           |
| HER2                    |           |           |                |           |           |                |
| Positive                | 20 (2.7%)  | 168 (20.3%) | 188 <0.0001   | HER2     | Positive   | 20 (9.6%)   | 113 (23.0%) | 133 <0.0001 |
| Negative                | 718 (97.3%) | 659 (79.7%) | 1377          | Negative  | 189 (90.4%) | 378 (77.0%) | 567           |
| Molecular subtypes      |           |           |                |           |           |                |
| Luminal A               | 467 (63.5%) | 126 (15.3%) | 593 <0.0001   |           |           |                |
| Luminal B               | 121 (16.5%) | 272 (32.9%) | 393           |           |           |                |
| HER2-enriched           | 10 (1.4%)  | 171 (20.7%) | 181           |           |           |                |
| Basal-like              | 24 (3.3%)  | 222 (26.9%) | 246           |           |           |                |
| Normal-like             | 113 (15.4%) | 35 (4.2%)  | 148           |           |           |                |

ER oestrogen receptor, PR progesterone receptor, LVI Lymphovascular invasion
improved by using the same definitions and evaluation methods for LVI status.

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AUTHOR CONTRIBUTIONS
S.K. participated in its design, experimentation, analysis, interpretation, and manuscript drafting. C.J., S.S., S.A., Y.K., A.A., M.A., M.A.A. and N.P.M. collected the genomic and clinical data and assisted in making the study design and evaluating the results obtained. L.D. mainly performed histopathological examinations. S.R., A.O., S.J., T.F., K.S., C.C., I.A., I.O.E., C.D., and A.R.G. contributed to theoretical organisation of the manuscript. E.A.R. conceived and supervised the study, participated in its design, interpretation, and analysis, including drafting. All authors contributed to drafting and reviewing the manuscript and approved the submitted and final version.

ADDITIONAL INFORMATION
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Competing interests: Ibraheem Alshankky is a consultant/advisory board in Molecular Diagnostics Lab, College of Applied Med. Sci., KAU. The remaining authors declare no competing interests.

Ethics approval and consent to participate: This study was approved by the Nottingham Research Ethics Committee 2 (Reference title: Development of a molecular genetic classification of breast cancer). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Data availability: The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Fig. 3 Survival analysis based on clinicopathological characteristics including LVI-related genomic subtype. Forest plots showing the hazard ratios and 95% CI of the multivariate survival analyses in a the METABRIC cohort and b the TCGA cohort. The LVI-related genomic subtype was an independent prognostic factor in both cohorts.
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