Frequent Molecular Subtype Switching and Gene Expression Alterations in Lung and Pleural Metastasis From Luminal A-Type Breast Cancer

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PURPOSE
Conversion of tumor subtype frequently occurs in the course of metastatic breast cancer but is a poorly understood phenomenon. This study aims to compare molecular subtypes with subsequent lung or pleural metastasis.

PATIENTS AND METHODS
In a cohort of 57 patients with breast cancer and lung or pleural metastasis (BCLPM), we investigated paired primary and metastatic tissues for differential gene expression of 269 breast cancer genes. The PAM50 classifier was applied to identify intrinsic subtypes, and differential gene expression and cluster analysis were used to further characterize subtypes and tumors with subtype conversion.

RESULTS
In primary breast cancer, the most frequent molecular subtype was luminal A (lumA; 49.1%); it was luminal B (lumB) in BCLPM (38.6%). Subtype conversion occurred predominantly in lumA breast cancers compared with other molecular subtypes (57.1% vs 27.6%). In lumA cancers, 62 genes were identified with differential expression in metastatic versus primary disease, compared with only 10 differentially expressed genes in lumB, human epidermal growth factor receptor 2 (HER2)–enriched, and basal subtypes combined. Gene expression changes in lumA cancers affected not only the repression of the estrogen receptor pathway and cell cycle–related genes but also the WNT pathway, proteinases (MME, MMP11), and motility-associated cytoskeletal proteins (CK5, CK14, CK17). Subtype-switched lumA cancers were further characterized by cell proliferation and cell cycle checkpoint gene upregulation and dysregulation of the p53 pathway. This involved 83 notable gene expression changes.

CONCLUSION
Our results indicate that gene expression changes and subsequent subtype conversion occur on a large scale in metastatic luminal A–type breast cancer compared with other molecular subtypes. This underlines the significance of molecular changes in metastatic disease, especially in tumors of initially low aggressive potential.

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INTRODUCTION
Visceral metastases often occur as late events in the course of metastatic breast cancer and are generally followed by a rapidly fatal outcome. Despite recent advances in our understanding of molecular events that occur during disease progression,1,2 details of the tumor biology of visceral metastases in comparison with primary breast cancer (PBC), and their relationship to tumor subtype, have not been fully elucidated. Molecular evolution is believed to be linked to the heterogeneity and molecular plasticity of primary breast cancer, which is reflected in gene expression.3 Lungs (23%) and pleura (12%) are the most common sites of visceral metastasis in breast cancer, followed by liver (10%) and brain (2%).4 In a large autopsy series, the incidence of lung metastases from breast primaries was as high as 71%.5 As a first site of tumor progression during the first 5 years of follow-up, the lungs rank third at 20%, after bone (38%) and liver (23%).6 When interpreting these figures, it must be noted that lung metastases are referred to as either including or excluding pleural metastasis. The site of metastasis in breast cancer is not random, but there is clinical and pathologic evidence of distinct patterns of disease relapse.7 Brain metastasis is often associated with the hormone receptor–negative/human epidermal growth factor receptor 2–positive (HER2-positive) phenotype, and liver metastasis also was more frequently observed in the HER2-positive subtypes compared with HER2-negative subtypes.8 In lung metastasis, there is no clear preference of a particular tumor subtype, but the luminal A subtype had a lower rate of lung relapse compared with the
other three subtypes by tissue microarray analysis, and basal subtypes in particular were more frequent than expected.

In this study, we included patients with invasive breast cancer of all molecular subtypes and metachronous breast cancer with lung or pleural metastasis (BCLPM) to provide insights into the up- and downregulation of genes during the course of the disease. Therefore, we retrospectively examined gene expression profiles in a well-characterized, prospectively assembled group of patients with breast cancer and metachronous lung or pleural metastasis (Data Supplement 2, Fig S1). Data were interpreted to provide information on which genes or gene groups undergo notable changes during the course of BCLPM regarding up- or downregulation.

PATIENTS AND METHODS

Study Population

Patient samples were recruited from a series of female patients with metastatic PBC and biopsy-confirmed BCLPM in 2003 to 2014. Patients had received both primary surgery and metastasis biopsies at the University Hospital, Heidelberg. Tumor histology of both sites and clinical records were reviewed (Data Supplement 2, Table S1), and pertinent data were updated retrospectively using current tumor classification criteria. No follow-up after metastasis was available.

Formalin-fixed and paraffin-embedded (FFPE) tissue samples were provided by the Tissue Bank at the National Center for Tumor Diseases (Heidelberg, Germany) in accordance with the regulations of the Tissue Bank and approval of the ethics committee of the medical faculty of the University of Heidelberg (approval No. S-716/2018). The final cohort of this study included 57 paired samples of PBC and BCLPM and was selected from 81 patients with BCLPM and PBC. Exclusion criteria included no availability or insufficiency of tumor tissue from PBC or BCLPM tumor tissue (n = 11), primary lung cancer after review of patient records (n = 2), and low RNA content or not meeting quality control criteria of RNA data (n = 11).

Gene Expression Analysis

For the selection of tumor tissue for RNA extraction, FFPE tissue blocks from the PBC and metastatic lesions were selected after reviewing all original tissue slides and were recut for hematoxylin & eosin sections, to be used for reference and to determine tumor cell content (tumor surface area). RNA was extracted using 5-10 unstained FFPE slides for each tumor. Microdissection was performed in most cases to avoid normal breast tissue contamination. A minimum of approximately 50 ng of total RNA was used. Hybridization time per cartridge was 16 hours before measurement.

RNA expression analysis was performed by measuring a custom panel of 269 breast cancer–related genes and 11 housekeeping genes (Data Supplement 2, Table S2), using the nCounter platform (Nanostring Technologies, Seattle CA) according to the manufacturer’s instructions. Two cases were excluded, because quality control criteria of RNA measurements were not met. The gene panel includes 25 published gene signatures with prognostic or predictive properties in luminal-type breast cancer with the aim to determine their role in metastatic disease (Data Supplement 2, Table S3). The selection of gene signatures covered by this list of genes includes nine proliferation-related signatures, four estrogen receptor–related signatures, one immune-related signature, and 11 signatures related to cancer pathways. In reporting this study, we have adhered to the recommendations for reporting tumor marker studies (REMARK guidelines).

Statistics

For molecular subtyping, the PAM50 subtype clustering model was fitted and risk of recurrence scores (ROR-S)
were calculated. Differential expression analysis was carried out using paired moderated $t$ statistic in limma, and nominal $P$ values were corrected for multiple comparisons using Benjamini and Hochberg’s method. All genes with an adjusted false discovery rate of $P < .05$ and a fold change of $< 0.66$ or $> 1.5$ were considered differentially expressed. For gene function, DAVID Bioinformatics Resources were used (version 6.8), and functional annotation analysis in the Biological Process category and KEGG pathway enrichment were computed. For two-dimensional visualization of the data, the UMAP method of multidimensional scaling was used. Time to metastasis was calculated using the Kaplan-Meier method. All statistical calculations, except gene function analysis, were done using R, version 3.6.1.

RESULTS

Patient Characteristics

A total of 57 patients with BCLPM were included in this study. The metastatic biopsy site was mostly pleura ($n = 48; 84.2\%$) and less often intrapulmonary ($n = 9; 15.8\%$). Three lung lesions presented as solitary metastasis, and 54 cases presented as multiple metastatic lesions. Kaplan-Meier analysis revealed significant differences for time intervals to BCLPM depending on the subtype of PBC (Data Supplement 2, Fig S2). The median interval between breast cancer diagnosis and BCLPM was 62.9 months.

PAM50 Subtypes of PBC and BCLPM

The most frequent subtypes were luminal A for PBC (49.1%) and luminal B for BCLPM (38.6%; Fig 1A). Two tumors with normal-like subtypes were identified in PBC, and none were identified in BCLPM. Tumor content in these samples was > 50%; therefore, these samples were regarded as representative, and neither case was excluded. A low-risk class was assigned to these cases, in concordance with the literature. When comparing molecular subtypes in PBC with BCLPM, discordant PAM50 (subtype switching) was observed in 24 cases (42.1%). PAM50 molecular subtype conversion occurred in the majority of luminal A-type PBC ($n = 16; 57.1\%$), evolving mostly into luminal B–type or HER2-type metastasis (Table 1; Fig 1B). In the other subtypes, a subtype switch occurred in only four of 14 luminal B–type tumors. Also, one HER2 enriched–type tumor recurred as luminal B metastasis, and one basal-like PBC recurred as a HER2-enriched subtype. Overall, subtype conversion resulted in increased numbers of high-risk subtypes in BCLPM versus PBC (73.7\% vs 47.4\%; $P = .007$; Data Supplement 2, Table S4).

PAM50 subtypes were clearly separated in UMAP multidimensional scaling on the basis of all 269 genes, and subtype mapping revealed differences in gene expression of PAM50 clusters with regard to primary or metastatic site. Gene expression clustering of BCLPM was clearly different from PBC in the low-risk category but not with cancers of high-risk subtypes (Figs 2A-2B). This indicates a similar tumor biology of BCLPM and PBC for tumors of high-risk subtypes but a different biology in the low-risk (luminal A) category. In particular, this method revealed that two distinct clusters of luminal A–type tumors were associated with either PBC or

![Fig 1](image-url)
## TABLE 1. Clinical and Molecular Characteristics of Luminal-Type Breast Cancers, Comparing LumA Subtypes With and Without Subtype Conversion to LumB-Type Tumors

| Characteristic | LumA Switched (n = 16) | LumA Not Switched (n = 12) | LumB (n = 14) |
|---------------|------------------------|-----------------------------|---------------|
| Median age, years (range) | 53.9 (33.4-67.5) | 55.6 (39.6-73.4) | 55.9 (27.3-76.0) |
| No. by tumor grade | | | |
| 1 | 1 (6.2) | 1 (8.3) | 0 (0) |
| 2 | 10 (62.5) | 11 (91.7) | 8 (57.1) |
| 3 | 2 (12.5) | 0 | 6 (43.1) |
| NA | 3 (18.8) | 0 | 0 |
| No. by TNM classification | | | |
| pT | | | |
| 1 | 8 (50.0) | 5 (41.7) | 3 (21.4) |
| 2 | 6 (37.5) | 1 (8.3) | 8 (57.1) |
| 3 | 0 (0) | 3 (25.0) | 1 (7.1) |
| 4 | 0 (0) | 0 (0) | 1 (7.1) |
| NA | 2 (12.5) | 3 (25.0) | 1 (7.1) |
| pN | | | |
| 0 | 5 (31.2) | 4 (33.3) | 4 (28.6) |
| 1 | 4 (25.0) | 1 (8.3) | 2 (14.3) |
| 2 | 1 (6.2) | 1 (8.3) | 4 (28.6) |
| 3 | 3 (18.8) | 3 (25.0) | 3 (21.4) |
| NA | 3 (18.8) | 3 (25.0) | 1 (7.1) |
| Median time to lung metastasis, months (range) | 118.4 (4.9-255.7) | 104.9 (38.6-255.0) | 56.2 (0.80-177.3) |
| Initial metastatic site before the treatment of metastatic breast cancer | | | |
| Lung or pleural metastasis | 7 (43.8) | 9 (75.0) | 10 (71.4) |
| Other | 8 (50.0) | 3 (25.0) | 4 (28.6) |
| Unknown | 1 (6.2) | 0 | 0 |
| Endocrine therapy at time of metastatic biopsy | | | |
| Yes, tamoxifen | 1 (6.2) | 0 | 0 |
| Yes, aromatase inhibitor | 2 (12.5) | 1 (8.3) | 3 (21.4) |
| Yes, GnRHa | 0 | 1 (8.3) | 0 |
| No | 13 (81.3) | 10 (83.3) | 11 (78.6) |
| No. of molecular subtype lung | | | |
| LumA | 12 (42.6) | 2 (14.3) | |
| LumB | 10 (35.7) | 10 (71.4) | |
| HER2 | 4 (14.3) | 1 (7.1) | |
| Basal | 2 (7.1) | 1 (7.1) | |
| No. of genes upregulated in lung metastases | 34 | 0 | 1 |
| Most significant genes (max, 10) | PTTG1, BUB1, MKI67, CENPA, PBK, CDC6, RRM2, TTK, CHEK1, DIAPH3 | None | ABI3BP |
| No. of genes downregulated in lung metastases | 49 | 7 | 1 |
metastatic breast cancer. Similarly, heatmap clustering confirmed separate luminal A clusters for primary tumors and metastases (Fig 3).

Differential Gene Expression Analysis
To characterize which genes are involved in the process of BCLPM, differential gene expression analysis was performed. For this purpose, a logistic model was fitted using a design matrix for paired samples comparisons between BCLPM and PBC. Across all subtypes, 41 genes were downregulated in BCLPM and 5 genes were upregulated (Data Supplement 2, Fig S3 and Table S5). Six genes were downregulated with a fold change of 0.25. These included 3 cytoskeletal proteins (KRT14, KRT17, KRT5), 2 matrix metalloproteinases (MMP11, MME), and ANKRD30A.

The latter is a breast differentiation gene that is frequently expressed in the breast and in receptor-positive breast cancer.22 Other molecular changes in metastases were linked to proliferation (CDC6, CCNB1, MKI67, TOP2A, AURKB, PTG1), cell cycle checkpoints (BRCA2, BUB1, BUB1B, CHEK1), and epithelial-mesenchymal transition (EMT) genes. Only one gene, SCRG1, was upregulated in BCLPM with a fold change of > 2, which is a marker of mesenchymal stem cells,23 pointing toward EMT in BCLPM. Among the genes most relevant for therapy in breast cancer (ESR1, PGR, HER2, MKI67), the expressions of ESR1 and HER2 were unaffected in BCLPM, but PGR was significantly downregulated (P = .0015, Wilcoxon rank-sum test), and MKI67 was not significantly increased (Data Supplement 2, Fig S5).

Subtype-Specific Changes
The PAM50 subtypes of PBC showed marked variation with regard to the extent of differential gene expression of this gene panel in BCLPM. The greatest number of differentially expressed genes was seen in luminal A–type breast cancer, with 47 and 11 down- and upregulated genes, respectively, compared with fewer expression changes in metastatic high-risk tumors (luminal B, HER2-enriched, basal-like), with six and four down- and upregulated genes, respectively (Figs 4A-4B). Only 1 gene was downregulated in BCLPM that was HER2 enriched, and no genes reached significance after adjustment for multiple testing in the basal-like subtype, which in part may be attributed to the comparably small sample sizes of 6 and 7, respectively (Data Supplement 2, Table S6). Of the genes repressed in luminal A metastases, several are associated with EMT. Four of these (TWIST2, TWIST1, ZEB1, TGFB3) are key regulators of EMT. In addition, STC2 promotes EMT via AKT and ERK, and PROM1 facilitates EMT.

Subtype Switching in Luminal A–Type Breast Cancers
Luminal A–type breast cancer demonstrated a unique behavior with regard to the frequency of subtype switching (Fig 1B), the phenotype in breast compared
with BCLPM (Figs 2A-2B), and the magnitude and type of gene expression changes in lung metastasis (Table 1). Thirty-four and 49 genes were significantly up- and downregulated, respectively, in BCLPM from luminal A cancers with subtype switch (adjusted $P < .05$) compared with only 7 genes downregulated and none upregulated in the non–subtype switch group (Figs 4C-4D). Interestingly, the time to progression to metastatic events was similar for tumors undergoing subtype switch or not (median, 118.4 v 104.9 months), suggesting that the events leading to higher-grade metastasis in luminal A cancers are late events in the metastatic process. In luminal A cancers, subtype switch affected cell cycle and cell proliferation genes (mitotic cell cycle checkpoint, sister chromatid cohesion, mitotic nuclear division, cell division) and the p53-signaling as well as progesterone-signaling pathways (Data Supplement 2, Figs 4A and 4B).

**Clustering of Gene Expression in Luminal A–Type Cancers**

To better characterize the phenomenon of subtype conversion in luminal A cancers, we set up a logistic model for the assessment of differential gene expression with and without subtype conversion. In the group of 28 luminal A–subtype tumors, a subtype conversion was more likely to occur when genes involved in certain growth factors, nuclear proteins, and signaling molecules were upregulated ($\text{GNAZ, HOXB13, VEGFA}$) and some genes involved in immune response and inflammation ($\text{FOXP3, CD8A, CD68, CSF1R}$) were downregulated (Fig 5A). In the metastatic setting, a much larger number of genes were clustered according to subtype switching of luminal A–subtype cancers. This included 10 genes of various functions with downregulation in metastases and 52 genes, also of diverse functions, that were upregulated after subtype switch (Fig 5B). This upregulation of a substantial number of genes suggests a common mechanism of gene regulation that is involved in subtype switching.

**Comparison of Gene Signatures in Luminal A– and B–Type Cancers**

For additional insight into the classes of genes involved in subtype switching, a couple of well-characterized gene signatures for estrogen receptor signaling, tumor proliferation, and hallmarks of cancer were analyzed in BCLPM (Data Supplement 2, Figs S6a and S6B). Interestingly, and despite the different genes involved in these signatures, all 4 tumor proliferation signatures tested showed similar patterns of gene expression in metastases derived from luminal A–type tumors with and without subtype switch. In particular, all proliferation signatures were significantly upregulated in subtype-switched tumors, with similar levels compared with luminal B cancers. Conversely, estrogen receptor–related signatures were downregulated in subtype-switched metastases of former luminal A–type breast cancers. However, no such changes in genes expression were seen in several gene signatures that are attributed to hallmarks of cancer.

**Data Availability**

The datasets generated and analyzed during this study are available in the GEO repository, GSE145752.

**DISCUSSION**

Metastatic breast cancer often belongs to a prognostically more aggressive category than the primary tumor. The molecular changes that are acquired by the tumor cell during disease progression have been summarized as molecular evolution and with respect to changes in
FIG 3. Unsupervised clustering heatmap of significantly differentially expressed genes in primary breast cancer and lung metastasis ($P < .05$, any fold change). Tumors are discriminated according to their molecular subtype, with separation of metastatic luminal A tumors from primary luminal A cancers (shown as blue bars across top). HER2, human epidermal growth factor receptor 2.

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intrinsic tumor subtype as subtype conversion or subtype switch.\textsuperscript{26} Our results indicate that evolution within cancer cell populations during metastasis leads to more transcriptomic and phenotypic changes than might be expected from the acquisition of genetic events. The magnitude of these changes and their relationships to molecular subtypes have not been studied systematically in visceral metastasis yet, but overall approximately 40% of organ metastases were shown to represent a different PAM50 subtype.\textsuperscript{27}

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**FIG 4.** Differential gene expression for breast cancer lung metastasis versus primary breast cancer. Genes with significant down- and upregulation are indicated in blue and red, respectively (adjusted \( P < .05 \)). Different patterns of gene expression changes are observed, with (A) significantly more changes in the luminal A subtype compared with (B) high-risk tumors (lumB, Human epidermal growth factor receptor 2 (HER2)–enriched, Basal-like). Within the luminal A subtype, gene expression changes were mostly confined to (C) tumors with subtype switch in lung metastasis compared with (D) nonswitched metastases.
FIG 5. Heatmaps of luminal A-type breast cancer genes ($P < .05$, any fold change) in breast cancer lung metastasis. Gene expression changes related to subtype switch are evident in both (A) primary breast cancers and (B) lung metastasis, each associated with alterations in various pathways, including proliferation-, estrogen receptor pathway-, and inflammation-associated genes.
Subtype conversion only partly reflects the changes occurring in tumor evolution, and our data indicate that, even without change of the PAM50 subtype, the molecular phenotype may still be different in the metastasis, especially in low-grade tumors. In multidimensional scaling, tumors of luminal A subtype in BCLPM form a different cluster than luminal A subtype in the primary (Fig 2), and, in the differential expression heatmap, they cluster with more aggressive subtypes (Fig 3). Along that line, relevant gene expression changes in brain metastases were found without change of PAM50 subtype.28 When PAM50 subtype conversion occurs, it can be regarded as an indicator of genetic remodeling in metastasis and as a risk factor of additional disease progression. This is also evident by the change in the risk of recurrence score, which almost doubled in luminal A-type cancers. Of note, although estrogen receptor and HER2 statuses as predictive clinical markers remained unchanged in this group, the proliferation rate greatly increased, and breast hormone receptor signaling declined. As such, although therapeutically relevant markers were unaltered upon subtype switch, remarkable changes occurred that are generally associated with a worsened malignant behavior and prognostic impact.

The gene expression changes that were evident across all subtypes included evidence for increased mobility and invasive behavior of the metastatic tumor cell through downregulation of cytokeratins (KRT5/14)29; tumor activation through downregulation of MMP1130,31; and deactivation of estrogen receptor–dependent pathways, as indicated by the downregulation of the progesterone receptor and the ankyrin repeat-domain gene (ANKRD30A). Conversely, genes involved in EMT and growth signaling were upregulated in all molecular subtypes (Data Supplement 2, Fig S3). As far as datasets are comparable, most changes found in BCLPM have been reported to occur also in other metastatic sites,27,32 with the exception of HER2, which was demonstrated to be upregulated in the brain28, however, it was not in our dataset.

Luminal A cancers undergoing a subtype switch were characterized by a much higher variability in gene expression in BCLPM compared with high-risk carcinomas. This variability affected various molecular pathways. From gene expression data alone, it appears not possible to name a common denominator for the changes in metastasis of luminal A cancers that affected several dozens of genes. When trying to dissect the alterations that occur in subtype conversion, we found that primary tumors with low expression of inflammation-related genes but increased expression of growth signaling–associated genes were more likely to undergo subtype conversion. In the metastatic tumor cell, the subtype switch was characterized by quite extensive gene expression changes of various molecular pathways, similar to the finding of important gene expression changes in > 100 genes in brain metastasis.29 No hypothesis about the root cause of changes for subtype conversion can be put forward, but the manifold changes of expression status in BCLPM from luminal A cancers do indicate a major reprogramming of the tumor cell.

Limitations of this study include the use of a curated gene list. As such, in contrast to genome-wide analyses, significant alterations in other, unrepresented pathways cannot be excluded in this study. Another limitation is that the selection of cases that underwent diagnostic biopsy was driven by clinical needs and may not be representative for metastatic breast cancer in general. Moreover, the molecular changes occurring in BCLPM were studied only on the RNA expression level, and no genomic assays were performed. Last but not least, although the discrimination of patients with breast cancer into specific cancer subtypes is a widely applied clinical concept with profound prognostic implications, luminal A and B cancers may much more represent a continuum rather than true distinct subtypes from a biologic point of view.

In conclusion, we have shown that luminal A–type breast cancer is most affected by subtype conversion and differential gene expression in matched metastases to lung or pleura involving but not limited to estrogen receptor signaling (downregulation) and tumor proliferation (upregulation). This supports additional molecular subtyping in luminal A–type breast cancer to better understand metastatic biology and clinical implications.

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST
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