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ARTICLE

Genomic profiling of metaplastic breast carcinomas reveals genetic heterogeneity and relationship to ductal carcinoma

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Abstract

Metaplastic breast carcinomas comprise a histologically heterogenous group of tumors. Although most are triple (estrogen/progesterone receptor, HER2) negative, these rare tumors are clinicopathologically distinct from other triple negative carcinomas and may be aggressive with worse chemotherapy responses. On the other hand, metaplastic carcinomas are histologically diverse, which is reflected in gene expression differences among subtypes. Whether metaplastic carcinomas are genetically distinct from other triple negative cancers and whether genetic differences underlie histologic subtypes remains poorly understood. We sequenced 408 cancer-related genes in 28 metaplastic carcinomas, including chondroid matrix-producing carcinomas ($n = 10$), spindle cell carcinomas ($n = 5$), and carcinomas with squamous ($n = 5$), mixed spindle/squamous ($n = 5$), and mixed metaplastic ($n = 3$) differentiation. Metaplastic carcinomas were highly enriched for *PIK3CA*/*PIK3R1* (61%) and Ras-Map kinase (25%) pathway aberrations compared to other triple negative carcinomas (TCGA dataset 14%, $p < 0.001$ and 7%, $p = 0.005$, respectively) and harbored a high frequency of *TP53* (64%) and *TERT* promoter (25%) mutations, but this varied among subtypes. Chondroid-matrix producing carcinomas lacked PI-3 kinase and Ras-Map kinase aberrations and *TERT* promoter mutations, compared to 100%, 39%, and 39% of non-matrix-producing tumors, respectively. *TERT* promoter mutations were enriched (47%) in spindle cell carcinomas and tumors with squamous or spindle/squamous differentiation. Spindle cell carcinomas lacked *TP53* mutations, in contrast to other subtypes (78%, $p = 0.003$). Separate analysis of paired ductal carcinoma in situ and metaplastic carcinoma revealed shared clonality in all cases ($n = 8$). Activating PI-3 kinase and Ras pathway mutations were early events, and inactivating mutations in tumor suppressors including *RB1*, *CDKN2A*, and *TP53* were associated with invasion in individual cases. Metaplastic components of two tumors showed genetic progression from separately sequenced paired invasive ductal carcinoma. The findings suggest that metaplastic carcinomas are genetically distinct from other triple negative breast cancers and highlight genetic heterogeneity that broadly correlates with histologic subtype. Heterologous elements progress from associated ductal carcinoma.

Introduction

Metaplastic breast carcinomas are a rare and histologically diverse group of breast cancers, estimated to comprise 0.2–5% of invasive carcinomas in the breast [1]. As a group, these tumors are defined histologically by differentiation of neoplastic epithelium into squamous or mesenchymal-like elements, including spindle cell and heterologous chondroid, osseous, or rhabdomyoid differentiation, among less common types [1–5]. A subgroup of cartilaginous and/or osseous matrix-producing carcinomas lacking a spindle cell component has been recognized as histologically distinct from other tumors with heterologous differentiation [5, 6]. The metaplastic component may arise with or without an associated in situ or invasive ductal component [7]. This heterogeneity is further highlighted by recognition of low-grade (fibromatosis-like and adenosquamous carcinoma) and more aggressive high-grade variants, which comprise the majority of tumors [1]. Most metaplastic breast carcinomas lack expression of estrogen

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receptor (ER), progesterone receptor (PR), and HER2 (triple negative phenotype), but are considered to have more aggressive behavior, worse outcomes, and poorer response to chemotherapy than other triple negative invasive ductal carcinomas of no special type [7–12]. Clinicopathologic features of metaplastic breast carcinomas are also distinct from other triple negative breast cancers, including presentation with larger tumor size, less frequent axillary lymph node involvement, and more common local recurrence/distant metastatic spread [7–9, 12, 13]. Histologic subtype has been identified in some studies as an independent prognostic feature [3–5, 7, 14]. Nonetheless, management and treatment of aggressive metaplastic breast carcinoma variants is typically similar between histologic subtypes and similar to other high grade triple negative carcinomas [2, 15].

The molecular features of metaplastic breast carcinomas are poorly defined, and the underlying basis for histologic heterogeneity remains uncertain. As a group, the majority of metaplastic breast carcinomas demonstrate basal-like or claudin-low gene expression profiles [16–19]. However, these tumors are transcriptomically heterogeneous within and between histologic subtypes. For instance, in contrast to other histologic subtypes, spindle cell carcinomas are uniformly claudin-low by expression profiling with lower expression of genes involved in epithelial-to-mesenchymal transition, whereas tumors with chondroid metaplasia are more homogeneously mesenchymal stem-like by triple negative breast cancer subtyping [17, 20]. Little is known regarding the genetics of metaplastic breast carcinomas. Some studies have demonstrated a high frequency of phosphoinositide (PI)-3 kinase pathway aberrations including frequent *PIK3CA* mutations, as well as *TP53* mutations, in a background of more heterogeneous lower frequency aberrations, such as *CDKN2A* loss, *EGFR* amplification, and Wnt pathway aberrations [18, 20–26]. Whether histologic subtypes are associated with distinct underlying genomic features and oncogenic driver aberrations compared to other subtypes remains largely unexplored but may help explain differences in behavior and outcome [3–5, 7, 14, 24]. A better understanding of pathogenesis and genetics may also lead to better treatment approaches for these aggressive tumors.

Although early studies demonstrated shared clonality of different histologic components in these tumors [27–31], the degree of genetic relatedness of different heterologous elements to one another and to associated invasive or in situ ductal carcinoma remains poorly characterized. In fact, the repertoire of somatic mutations of ductal carcinoma in situ (DCIS) associated with metaplastic carcinomas has not been investigated and could shed light on tumor histogenesis.

In this study, we used capture-based next-generation sequencing of 408 cancer-related genes in order to comprehensively characterize the genomics of metaplastic breast carcinomas and to compare the genetics of various histologic subtypes with one another and with publically available data of other triple negative breast cancers. Metaplastic components in a subset of tumors were also compared with associated in situ and invasive ductal carcinoma in order to determine the genetic relationship between these components. The findings shed light on our understanding of the biology and progression of metaplastic breast carcinomas and highlight the underlying genetic heterogeneity of these rare aggressive tumors.

Materials and methods

Study population

This study was approved by the institutional review board of the University of California San Francisco. Twenty-eight metaplastic breast carcinomas, spanning years 2003–2017, were identified in the Pathology department archives. Low grade fibromatosis-like and adenosquamous carcinomas were excluded. All cases were reviewed by G.K. and Y-Y.C to confirm the diagnosis and lines of differentiation. All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Clinical information was obtained from the electronic medical record of UCSF or referring facilities when applicable.

Capture-based next generation DNA sequencing

Matched normal and tumor tissue was selected from 23 metaplastic carcinomas for capture-based next-generation DNA sequencing. For an additional five cases (Sp2, Sp3, Sq3, Sq5, and SS2), only tumor tissue was selected for sequencing. For eight tumors, DNA from associated DCIS was extracted and analyzed separately. For three tumors (including two for which DCIS was analyzed separately), DNA was extracted and analyzed from two separate areas with differing lines of differentiation. Sequencing libraries were prepared from genomic DNA extracted from tumor and normal formalin-fixed paraffin embedded tissue. Target enrichment was performed by hybrid capture using a custom oligonucleotide library. Sequencing was performed at the UCSF Clinical Cancer Genomics Laboratory using an assay that targets the coding regions of 408 cancer-related genes, select introns from 42 genes, and *TERT* promoter with a total sequencing footprint of 2.8 Mb (Supplemental Table S1) [32, 33]. Sequencing was performed on an Illumina HiSeq 2500. Duplicate sequencing reads were removed computationally to allow for accurate allele frequency determination and copy number calling. The analysis was based on the human reference sequence UCSC build hg19 (NCBI build

37), using the following software packages: BWA: 0.7.10-r789, Samtools: 1.1 (using htslib 1.1), Picard tools: 1.97 (1504), GATK: 2014.4-3.3.0-0-ga3711, CNVkit: 0.3.3, Pindel: 0.2.5a7, SATK: 2013.1-10- gd6fa6c3, Annovar: v2015Mar22, Freebayes: 0.9.20 and Delly: 0.5.9 [34–44]. Only insertions/deletions (indels) up to 100 base pairs in length were included in the mutational analysis. Somatic single nucleotide variants and insertions/deletions were visualized and verified using Integrated Genome Viewer. Genome-wide copy number analysis based on on-target and off-target reads was performed by CNVkit and Nexus Copy Number (Biodiscovery, Hawthorne, CA) [38]. Tumors treated with neoadjuvant chemotherapy prior to sequencing were excluded from copy number analysis.

Results of DNA sequencing were compared to publicly available sequencing data from breast carcinomas in The Cancer Genome Atlas (TCGA) sorted for triple negative status [45, 46]. Statistical comparisons between metaplastic carcinomas and TCGA breast cancers and between metaplastic carcinoma histologic subtypes were performed using Fisher exact, chi-squared, and student's *t*-tests using a level of significance of $p < 0.05$.

Immunohistochemistry

Immunohistochemistry for ER, PR, and HER2 was performed on whole tissue sections using CC1 (Roche, Ventana Medical Systems) for antigen retrieval and clones SP1, 1E2, and 4B5, respectively (all undiluted; Roche, Ventana Medical Systems, Tucson, AZ). Positive staining was defined according to ASCO/CAP guidelines.

Results

Clinicopathologic features of metaplastic carcinomas

Clinicopathologic features of metaplastic carcinomas included in this study are shown in Table 1. Patient ages ranged from 25 to 85 years (mean 61, median 64 years). Cases included five pure spindle cell carcinomas (Sp1-5), five carcinomas with squamous differentiation (Sq1-5), five carcinomas with mixed spindle and squamous differentiation (SS1-5), ten matrix-producing carcinomas with chondroid differentiation, one of which also had a minute focus (<5%) of associated bone (C1-10), and three carcinomas with mixed heterologous differentiation (M1-3) (Figs. 1 and 2, Supplemental Table S2). Chondroid matrix-producing tumors were nodular with circumscribed borders and composed of carcinoma cells directly embedded within cartilaginous-like matrix without an associated spindle cell component (Fig. 1 and Supplemental Table S3) [5]. Pure spindle cell carcinomas were entirely sarcomatoid and lacked conventional ductal, squamous, or other heterologous elements. All tumors designated as matrix-producing, squamous, spindled, or squamous/spindled lacked differentiation along other metaplastic lineages. Twenty-one (75%) tumors were associated with a ductal component, including fifteen (54%) with invasive ductal carcinoma and twelve (43%) with DCIS (Table 1 and Supplemental Table S2). Aside from two pure spindle cell carcinomas (Sp2 and Sp3), all tumors showed features of modified Scarff-Bloom-Richardson grade 3. When present, DCIS was high nuclear grade in all but two cases and was predominantly solid and comedo patterns (Table 1). Ninety-three percent (26/28) of tumors were triple negative; one mixed spindle and squamous carcinoma (SS4) was HER2-positive in the squamous but not the spindle cell component, and one squamous cell carcinoma (Sq5) was ER-positive. Nine (32%) tumors had axillary lymph node metastases, of which five (56%) were of mixed squamous and spindle cell or squamous cell histology. Of patients with clinical follow-up ($n = 25$; mean 31, range 3–167 months), distant metastases were only identified in primary tumors with mixed heterologous components (M1–M3), and all three were metastatic (Table 1).

Genomics of metaplastic carcinomas and correlation with histologic subtype

Lineage differentiation of the sequenced and non-sequenced invasive tumor components is shown in figure 2. The mean target sequencing coverage was 440 (±250) unique reads per target interval (mean 474 ± 256 for invasive cancer, 306 ± 178 for DCIS) (Supplemental Table S4). The number of identified nonsynonymous coding mutations across the 2.8 megabase footprint of the panel was highly variable and ranged from 1 to 51 per invasive tumor (mean 9.2 ± 9.6), without differences across subtypes (Fig. 2 and Supplemental Table S5). The most frequently identified pathogenic aberrations were in PI-3 kinase pathway genes and in *TP53*, which were each altered in 64% (18/28) of tumors. Sixty-one percent (17/28) of all tumors harbored either *PIK3CA* activating mutations/amplification or *PIK3R1* inframe indels/truncating mutations, and this was dependent on histologic subtype (Figs. 2 and 3). No chondroid matrix-producing tumors harbored mutations in *PIK3CA* or *PIK3R1* or in other PI-3 kinase pathway genes ($p < 0.001$ vs. other subtypes). In contrast, all other (squamous, squamous/spindled, spindle cell, and mixed) metaplastic carcinomas had PI-3 kinase pathway aberrations, including mutations in *PIK3CA* or *PIK3R1* in 17 of the 18 (94%) tumors. The only non-matrix-producing tumor lacking mutations in *PIK3CA* or *PIK3R1* (squamous cell carcinoma Sq5) harbored an activating mutation (p.E17K) in the

Table 1 Clinicopathologic features of metaplastic carcinomas

Case	Age/ gender	Differentiation	Size (cm)	Grade	ER/PR/ HER2	DCIS	DCIS grade/pattern	Lymph nodes	Distant mets
Sp1	82/F	Spindle	3	3	−/−/−	Yes	Intermediate/micropapillary, papillary, cribriform	0/2	No
Sp2	85/F	Spindle	4.8	2	−/−/−	No	–	NA	No
Sp3	67/F	Spindle	2.5	2	−/−/−	No	–	0/1	No
Sp4	45/F	Spindle	11.4/12[b]	3	−/−/−	No	–	4/41	No[c]
Sp5	56/F	Spindle	2.3	3	−/−/−	Yes	High[d]/solid, cribriform, comedo	0/3	No
SS1	61/F	Squamous and spindle	10	3	−/−/−	No	–	0/4	No
SS2	79/F	Squamous and spindle	6.3	3	−/−/−	No	–	NA	NA
SS3	82/F	Squamous and spindle	1.4	3	−/−/−	Yes	High/ solid, cribriform, comedo	1/4	No
SS4	60/F	Squamous and spindle	6.4/1.6[b]	3	−/−/+	Yes	High/solid	1/15	No
SS5	57/F	Squamous and spindle	2.2	3	−/−/−	No	–	1/14	No
Sq1	64/F	Squamous	13/12[b]	3	−/−/−	Yes	High/solid	9/16	No
Sq2	69/F	Squamous[a]	5.1	3	−/−/−	Yes	High/micropapillary, comedo	2/49	No
Sq3	63/F	Squamous	4.4	3	−/−/−	Yes	High/solid, micropapillary, comedo	0/1	No
Sq4	65/F	Squamous[a]	6.6	3	−/−/−	Yes	High/solid, comedo	0/4	No
Sq5	54/F	Squamous[a]	3.2	3	+/−/−	Yes	High/solid and comedo	0/2	No
C1	71/F	Chondroid[a]	2	3	−/−/−	No	–	0/1	No
C2	45/F	Chondroid[a]	3/1.7[b]	3	−/−/−	No	–	1/7	No
C3	52/F	Chondroid[a]	4.7	3	−/−/−	No	–	3/9	No
C4	78/F	Chondroid[a]	1.4	3	−/−/−	No	–	0/1	No
C5	25/F	Chondroid[a]	2.5	3	−/−/−	No	–	0/7	No
C6	42/F	Chondroid[a]	1.8	3	−/−/−	Yes	High/solid, comedo	0/1	No
C7	26/F	Chondroid[a]	2.7/4.8[b]	3	−/−/−	No	–	0/1	No
C8	75/F	Chondroid[a]	1.5	3	−/−/−	No	–	0/3	No
C9	73/F	Chondroid[a]	4	3	−/−/−	Yes	Intermediate/solid	0/2	No
C10	74/F	Chondroid, focal osseous[a]	0.9	3	−/−/−	Yes	High/solid	0/1	No
M1	70/F	Spindle, squamous, osseous	6	3	−/−/−	No	–	0/1	Lung, (osseous)
M2	46/F	Spindle, osseous, chondroid, rhabdomyoid[a]	NA/16.5[b]	3	−/−/−	No	–	2/6	Intercostal
M3	50/F	Squamous, chondroid, osseous, spindle[a]	2.7/10[b]	3	−/−/−	No	–	0/8	Lung (squamous)

[a]Also with ductal component

[b]Imaging size before neoadjuvant chemotherapy/pathologic size after neoadjuvant chemotherapy

[c]Local chest wall recurrence

[d]Apocrine ductal carcinoma in situ

downstream kinase *AKT1* (Fig. 2 and Supplemental Table S5). Pathogenic Ras-Mitogen activated protein (Map) kinase pathway aberrations were identified in 25% (7/28) of tumors and included activating variants of *KRAS* ($n = 2$) and *HRAS* ($n = 2$), deep deletions of *NF1* ($n = 2$), and a *MAP2K1* missense hotspot mutation (Figs. 2 and 3, Supplemental Table S5). Similar to PI-3 kinase pathway aberrations, Ras-Map kinase pathway mutations were not identified in chondroid matrix-producing tumors (0/10 vs. 7/18 [39%] other subtypes, $p = 0.03$) (Figs. 2 and 3). Hotspot *TERT* promoter mutations were identified in 39% (7/18) of non-chondroid tumors, including 4/5 spindle cell

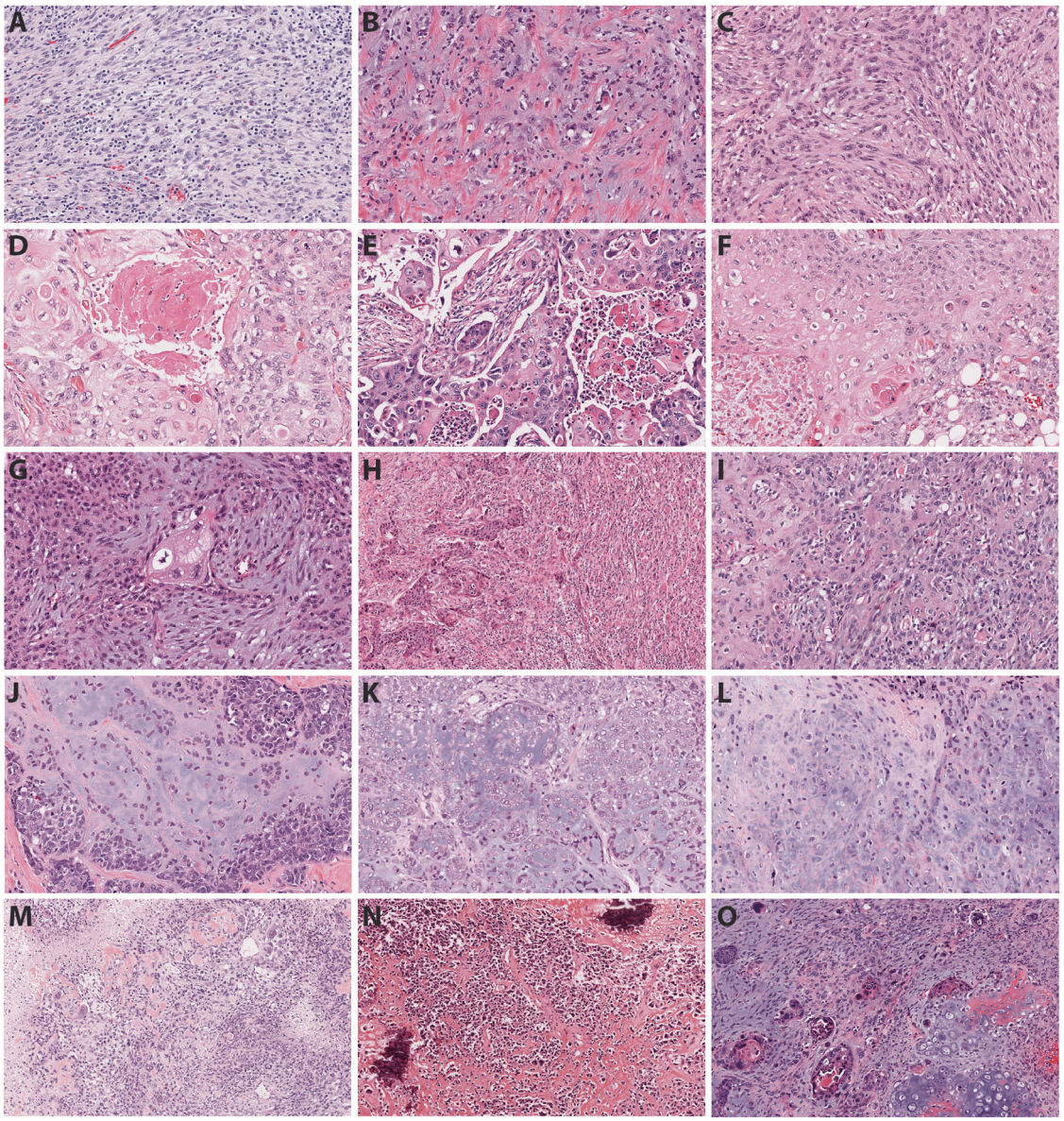


Fig. 1 Representative images of metaplastic breast carcinomas. **a–c** Pure spindle cell carcinomas were entirely composed of spindled tumor cells without other heterologous or conventional ductal elements (Sp1, Sp2, and Sp4, respectively). **d–f** Metaplastic carcinomas with squamous differentiation (Sq1, Sq3, and Sq4, respectively). **g–i** Metaplastic carcinomas with mixed squamous and spindle cell differentiation (SS1, SS3, and SS4, respectively). **j–l** Matrix-producing metaplastic carcinomas with chondroid differentiation (C1, C6, and C7, respectively). Carcinoma cells directly transition to tumor cells embedded within chondroid matrix without associated spindled tumor cells or other heterologous components. **m–o** Metaplastic carcinomas with mixed heterologous differentiation (M1, M2, and M3, respectively). These tumors demonstrated admixed areas of differentiation along multiple heterologous lineages. Areas containing osseous differentiation were sequenced and are shown here, along with foci of spindled tumor cells (*m-o*), chondroid (*o*), and squamous (*o*) elements. All images hematoxylin and eosin

carcinomas, 2/5 mixed spindled/squamous cell carcinomas, and 1/5 squamous cell carcinomas. The C228T (chr5: 1295228) variant was identified in 6/7 tumors, whereas one squamous cell carcinoma harbored the C250T (chr5: 1295250) variant (Supplemental fig S5). No *TERT* promoter mutations were identified in chondroid matrix-producing carcinomas (0/10, 0%; $p = 0.03$) (Figs. 2 and 3). These tumors were instead enriched for *TP53* mutations (90%) but were otherwise more heterogeneous, with inactivating mutations in tumor suppressors such as *NOTCH1*, chromatin remodeling genes (*ARID1A*, *KMT2A*, *KMT2D*), and DNA repair genes (*ERCC2*, *PALB2*) (Fig. 2 and Supplemental Table S5). None of the pure spindle cell carcinomas (0/5) harbored *TP53* mutations, in contrast to the high

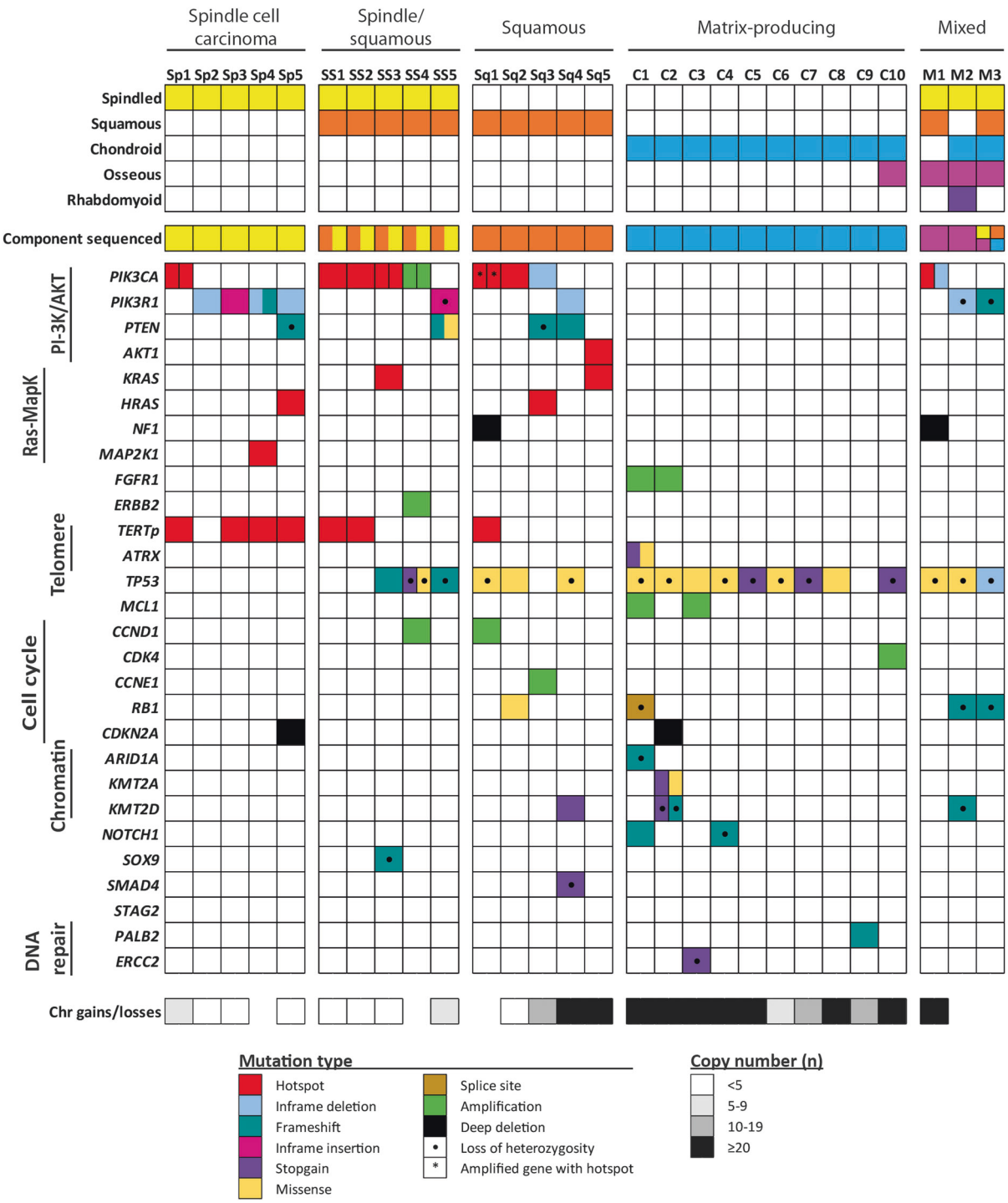


Fig. 2 Summary of pathogenic genomic aberrations in metaplastic carcinomas

frequency (18/23, 78%) identified across the other histologic groups ($p = 0.003$) (Figs. 2, 3). The genetic profiles of tumors with mixed heterologous differentiation in which the osseous component was sequenced were overall similar to other non-matrix-producing tumors and included pathogenic *PIK3CA*/*PIK3R1* and *TP53* mutations in all cases (Figs. 2, 3).

Copy number analysis revealed variable numbers of chromosomal copy number gains and losses between and within histologic subgroups, overall ranging from 0 to 51

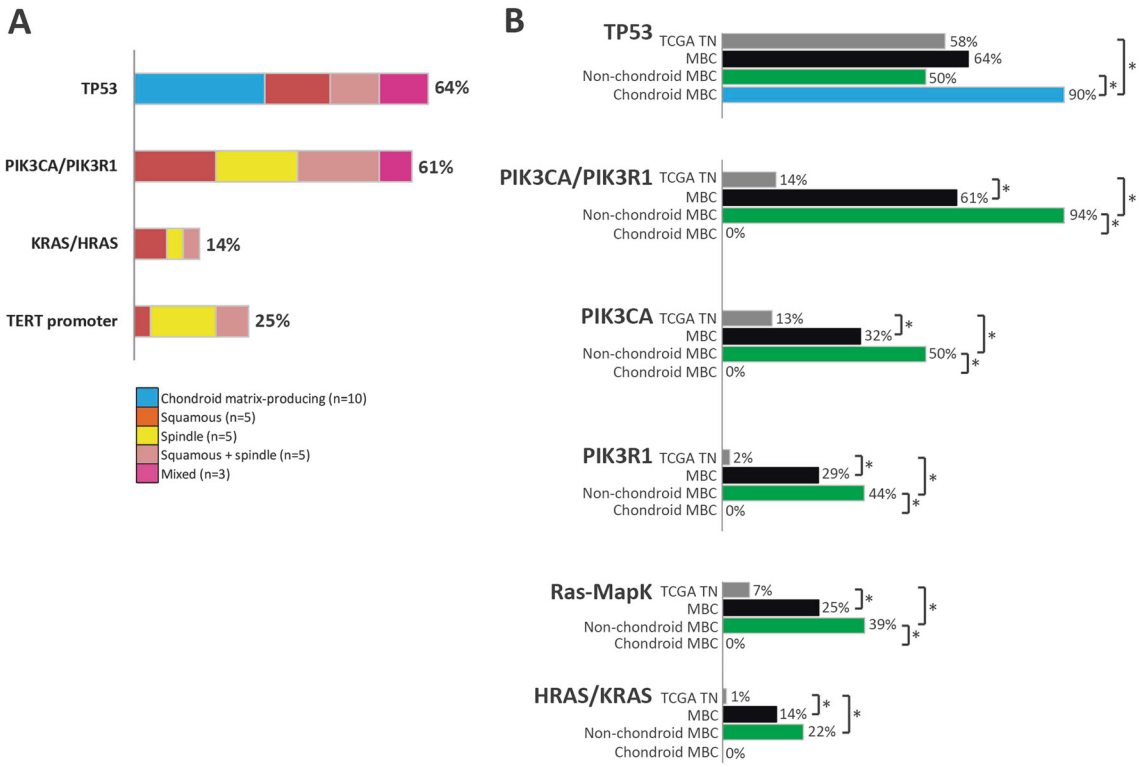


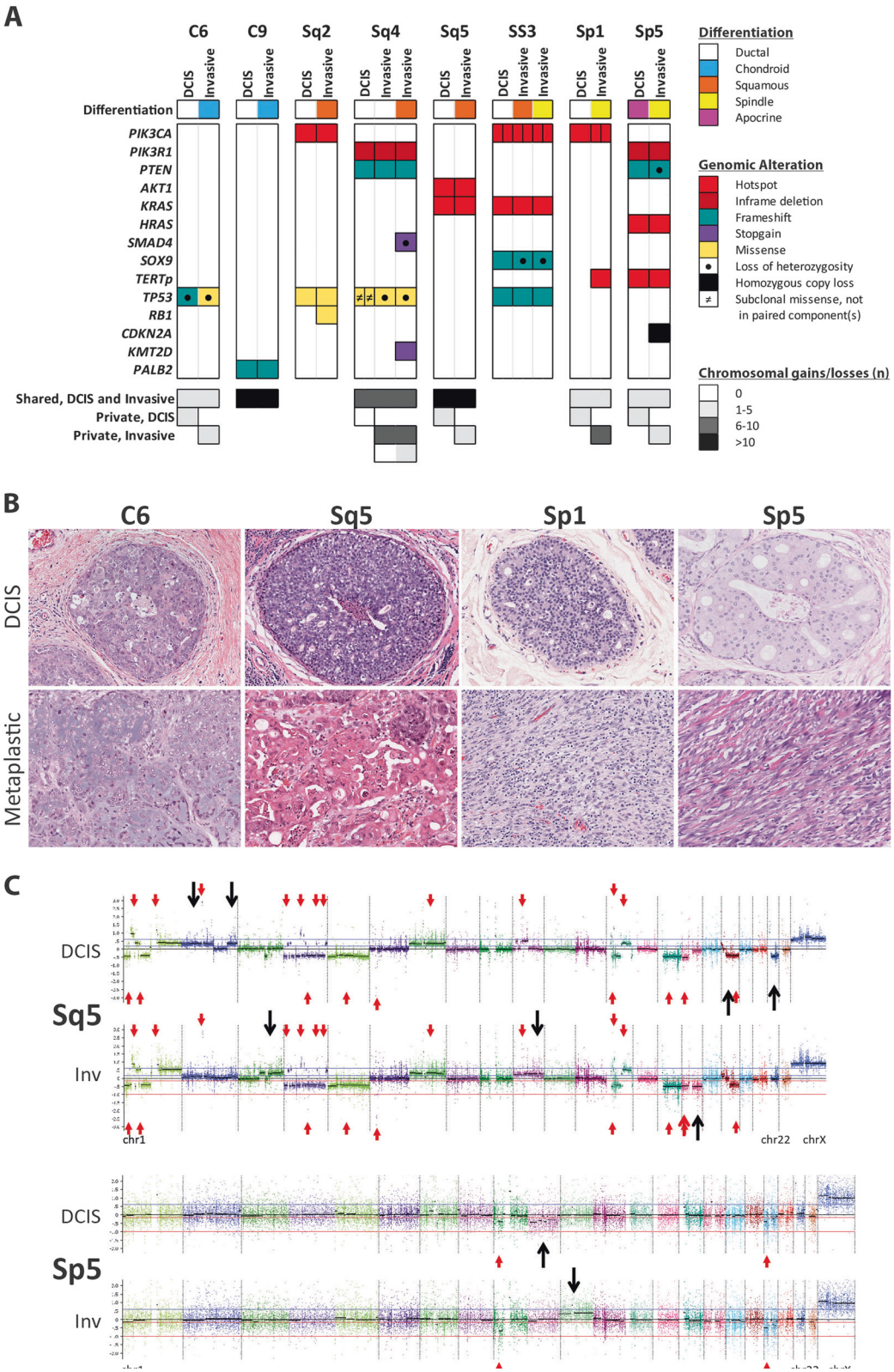
Fig. 3 Genetic heterogeneity of metaplastic carcinoma histologic subtypes with comparison to triple negative carcinomas of no special type. **a** Frequency of genetic pathway aberrations between histologic subtypes. Percentages reflect total relative number of tumors with designated mutation(s). **b** Comparison of genetic pathway aberrations between metaplastic carcinomas and triple negative carcinomas from the TCGA dataset [45, 46]. TCGA, The Cancer Genome Atlas; TN, triple negative; MCB, metaplastic breast carcinoma; $*p < 0.05$

alterations per tumor (mean 12.6 ± 10.1). Chondroid matrix-producing carcinomas had higher overall numbers of chromosomal copy number changes (mean 23.1 ± 10.9) than the other subtypes (mean 7.7 ± 9.6, $p = 0.002$), with mixed squamous/spindle cell and pure spindle cell carcinomas each showing few copy number alterations (mean 1.5 ± 3 and 2.8 ± 3.1, respectively; $p < 0.005$ each vs. chondroid matrix-producing tumors). Tumors with squamous differentiation also showed high numbers of chromosomal alterations (mean 14 ± 9.5, range 0–20), similar to chondroid matrix-producing carcinomas ($p = 0.171$ vs. chondroid matrix-producing, $p = 0.046$ vs. squamous/spindle, and $p = 0.06$ vs. spindle cell carcinomas) (Supplemental Table S6).

Comparing genomic profiles of metaplastic carcinomas with other triple negative breast carcinomas

We compared the genomic profiles of metaplastic carcinomas in our study population to publicly available sequencing data of triple negative breast cancers ($n = 125$) analyzed in the TCGA (Fig. 3) [45, 46]. The frequency of *TP53* mutations was overall comparable between metaplastic carcinomas and TCGA triple negative tumors (64% vs. 58%, respectively), but *TP53* mutations were more common in chondroid matrix-producing carcinomas (90%, $p = 0.05$). Metaplastic carcinomas as a group more frequently harbored *PIK3CA* (32% vs. 13%, $p = 0.021$) or *PIK3R1* (29% vs. 2%, $p < 0.001$) mutations than triple negative TCGA tumors, with 61% of metaplastic carcinomas having pathogenic aberrations in either gene, compared to only 14% of TCGA triple negative tumors ($p < 0.001$). The differences were even more striking when matrix-producing carcinomas were excluded (50% *PIK3CA*, 44% *PIK3R1*, and 94% *PIK3CA* or *PIK3R1* mutations in non-matrix-producing tumors, $p < 0.001$ each vs. TCGA). No significant differences were identified between metaplastic carcinomas and TCGA triple negative tumors in other PI-3 kinase pathway genes, such as *PTEN* (14% vs. 7%, $p = 0.258$) or *AKT1* (1 vs. 4%, $p = 0.334$). Metaplastic carcinomas also had more frequent Ras-Map kinase pathway genetic aberrations than TCGA triple negative tumors (25 vs. 7%, respectively, $p = 0.005$), a difference which was further amplified in non-matrix-producing tumors (39%, $p < 0.001$). This increased frequency in Ras-Map kinase pathway mutations was due to more activating *HRAS* or *KRAS* mutations in metaplastic carcinomas (14% in all

tumors, 22% in non-chondroid matrix tumors) compared to TCGA triple negative tumors, in which such mutations are rare (1%, $p = 0.004$). The results were similar when excluding the rare ER+ (Sq5) and HER2+ (SS4) metaplastic carcinomas from analysis (data not shown).



◀ **Fig. 4** Genomic aberrations in paired metaplastic carcinomas and associated ductal carcinoma in situ. **a** Summary of pathogenic genomic aberrations in paired invasive and in situ carcinomas. **b** Representative images of paired ductal carcinoma in situ and invasive metaplastic carcinomas of different histologic subtypes (hematoxylin and eosin). **c** Representative copy number plots of paired ductal carcinoma in situ and invasive metaplastic carcinoma from tumors with squamous (Sq5) and spindle cell (Sp5) differentiation. Small red arrows highlight shared copy number alterations between in situ and invasive carcinoma, and large black arrows highlight alterations unique to only one of the two components per tumor. DCIS, ductal carcinoma in situ; Inv, invasive; chr, chromosome

Genetic relationship of metaplastic carcinomas with paired invasive and in situ ductal carcinoma

Ductal carcinoma was microdissected and sequenced separately from the invasive heterologous components of nine metaplastic carcinomas, including paired DCIS of eight tumors (C6, C9, Sq2, Sq4, Sq5, Sp1, Sp5, and SS3) and paired invasive ductal carcinoma of two tumors with either chondroid matrix-production (C1) or squamous (Sq4) differentiation (Figs. 4, 5). Of the eight tumors in which paired DCIS was sequenced, two were pure spindle cell carcinomas (Sp1 and Sp5), and DCIS in one of these (Sp5) was apocrine type. DCIS in cases of chondroid matrix-producing (C6 and C9), squamous (Sq2, Sq4, and Sq5) or mixed squamous/spindle cell (SS3) carcinomas was of no special histologic type. In all cases, DCIS and invasive heterologous components were found to be genetically related, as determined by shared single nucleotide variants/indels (7/8 cases) and/or shared copy number changes (6/6 cases) (Fig. 4, Supplemental Tables S5 and S6). Pathogenic activating mutations in PI-3 kinase or Ras pathway genes were present in both DCIS and invasive cancer in all paired cases harboring these alterations ($n = 6$), indicative of an early event in tumorigenesis. In contrast, inactivating mutations in tumor suppressor genes such as *RB1* (Sq2), *CDKN2A* (Sp5), *SMAD4* (Sq4), and *KMT2D* (Sq4) were associated with stromal invasion and/or tumor progression (Fig. 4). The lack of these mutations in DCIS could not be explained by differences in tumor cellularity, depth of coverage, or mutant allele frequencies between the components (Supplemental Tables S4 and S5). In two cases (Sq2 and SS3), shared *TP53* mutations were identified in the paired DCIS and invasive components, whereas two other tumors (C6 and Sq4) harbored different *TP53* mutations in DCIS and paired invasive cancer, with the DCIS-associated variants present at subclonal mutant allele frequency (Fig. 4). The findings in the latter tumors support intratumoral heterogeneity of *TP53*-mutant clones in DCIS with outgrowth of a dominant clone upon progression to invasion. In addition to shared copy number changes between the in situ and invasive components in all informative tumors, private copy number changes were also identified in the DCIS of 4/6 cases and in the invasive components of 5/6 cases (Fig. 4).

In both tumors in which the invasive ductal and heterologous components were separately sequenced (C1 and Sq4), the paired elements were genetically related to one another, as determined by multiple shared single nucleotide variants/indels and copy number changes (Fig. 5, Supplemental Tables S5 and S6). The chondroid component of tumor C1 and the squamous component of tumor Sq4 each harbored additional clonal pathogenic mutations (in *ATRX*/*RB1*/*ARID1A* and *SMAD4*/*KMT2D*, respectively) that were not present in the respective paired ductal carcinoma components, and the number of private variants of unknown significance was higher in heterologous components than paired ductal carcinoma ($n = 43$ vs. 11 in tumor C1 and $n = 11$ vs. 4 in tumor Sq4, respectively) (Fig. 5 and Supplemental Table S5). The heterologous components of tumors C1 and Sq4 also harbored more chromosomal copy number changes than paired ductal components (Supplemental Table S6). Analysis of separately sequenced DCIS, invasive ductal, and invasive squamous components in squamous/ductal tumor Sq4 was particularly illustrative for detailing genetic relatedness and tumor progression (Fig. 5). In this tumor, inframe *PIK3R1* (p.579_582del) and frameshift *PTEN* (p.Y240fs) mutations were identified as early events present in all three components, whereas a clonal missense *TP53* mutation (p.G266R) was acquired during progression to invasion and shared among the invasive components but not DCIS. The invasive squamous component additionally harbored clonal inactivating *SMAD4* (Q334*) and *KMT2D* (S2927*) mutations, as well as numerous variants of unknown significance not present in either *in situ* or invasive ductal carcinoma (Fig. 5).

In contrast to the results of metaplastic tumors with ductal components, separately sequenced squamous and spindle cell components of a mixed squamous/spindle cell carcinoma (SS3) showed no pathogenic genetic differences between heterologous components (Fig. 4 and Supplemental Table S5).

Discussion

The clinicopathologic, histopathologic, and molecular heterogeneity of triple negative breast cancers is well established [47–49]. We show that metaplastic breast carcinomas comprise a genetically distinct subgroup of triple negative tumors with a high frequency of PI-3 kinase and Ras-Map kinase pathway aberrations compared to other triple negative breast cancers. Previous studies have also demonstrated PI-3 kinase pathway alterations in metaplastic carcinomas but were limited by lack of histologic annotation, inclusion of mixed tumors, inclusion of only sarcomatoid or

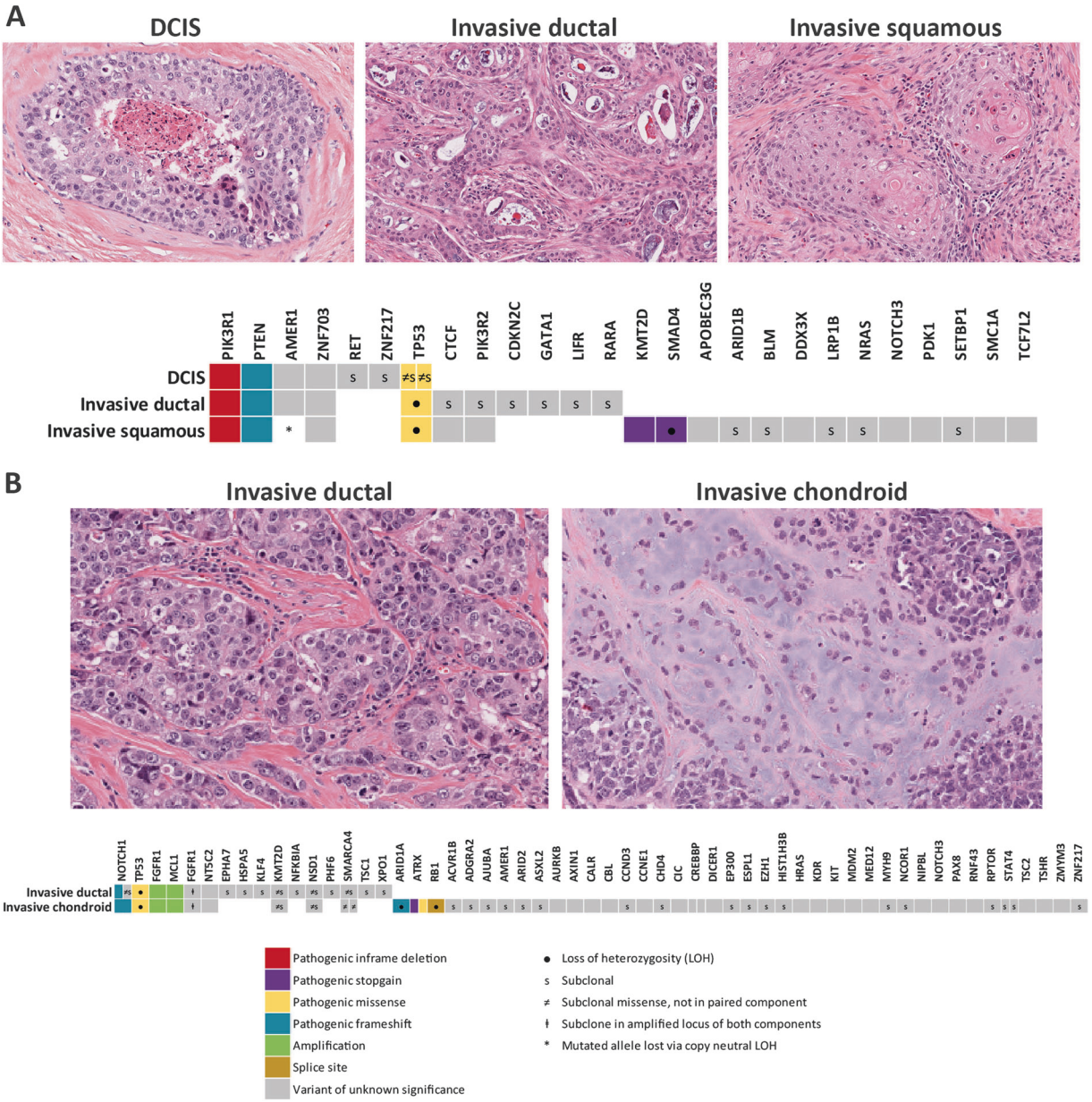


Fig. 5 Genetic progression of metaplastic carcinomas from ductal to heterologous differentiation. **a** Paired ductal carcinoma in situ, invasive ductal, and invasive squamous components of tumor Sq4 were microdissected and sequenced separately. Clonal *PIK3R1* (p.579_582del), *PTEN* (p.Y240fs), and *ZNF703* (p.179_181del) mutations were shared between all three components. A clonal pathogenic *TP53* missense mutation (p.G266R) was shared only between invasive ductal and squamous components, whereas the sampled in situ component harbored two different *TP53* missense mutations (p.K132N, p.R213L) at subclonal allele frequencies that were not present in the invasive cancer. Histologic progression to squamous cell carcinoma was associated with unique truncating pathogenic *KMT2D* (p.S2927*) and *SMAD4* (p.Q334*) mutations. **b** Paired invasive ductal and invasive chondroid components of tumor C1 were microdissected and sequenced separately. *FGFR1* and *MCL1* amplifications, as well as frameshift *NOTCH1* (p.P2514fs), missense *TP53* (p.V173M), and missense *NT5C2* (p.D306G) mutations, were shared between components. Pathogenic *ATRX* (p.E873*), *RB1* (c.1128-2 A > G), and *ARID1AI* (p.E1904fs) mutations were unique to the chondroid component, along with numerous additional variants of unknown significance at clonal and subclonal allele frequencies. DCIS, ductal carcinoma in situ

squamous variants, or analysis of advanced stage treated tumors [18, 22, 23, 26]. Our data indicate that metaplastic carcinomas are genetically heterogeneous and that this correlates broadly with histologic subtype. Chondroid matrix-producing carcinomas conspicuously lack *PIK3CA/PIK3R* and Ras pathway aberrations, which are very common in other subtypes, including tumors with squamous and/or spindle cell differentiation and tumors with mixed heterologous elements. In this context, our findings expand on those of Ng et al. [24], who also described a lack of

PIK3CA mutations in tumors with chondroid metaplasia, although whether the tumors in their study were strictly defined matrix-producing carcinomas is unclear [5]. We suggest that matrix-producing carcinomas are not only histologically but also genetically distinct from other metaplastic carcinomas, including those with foci of chondroid metaplasia in a mixed heterologous background, supporting classification as a distinct subgroup. Enrichment of PI-3 kinase aberrations only in non-matrix producing carcinomas may have treatment implications, as the demonstrated clinical efficacy of PI-3 kinase inhibitors in metaplastic carcinomas and triple negative tumors of mesenchymal molecular subtype correlates with PI-3 kinase pathway mutations [21, 23]. This raises the consideration that matrix-producing tumors may be less likely than other histologic variants to respond to PI-3 kinase pathway inhibition, which will require further study.

Hotspot *TERT* promoter mutations, which upregulate telomerase reverse transcriptase (TERT) expression and activate telomerase, are among of the most frequently identified mutations in certain tumors but have been considered to be absent or exceedingly rare in carcinomas of the breast [50–53]. A recent study addressing this issue identified *TERT* promoter mutations in only 3 (0.9%) of 319 invasive breast cancers, two of which were ER/PR-positive and one of which was triple negative [53]. Other groups have not found *TERT* promoter mutations in several breast cancer cohorts [51, 52]. We demonstrate for the first time a high frequency of hotspot *TERT* promoter mutations in metaplastic breast carcinomas and specifically in tumors with spindle cell and/or squamous differentiation (39%) but not matrix-producing carcinomas, which highlights the importance of telomerase activation in the former subgroups. *TERT* promoter mutations have been associated with poor clinical outcome in several tumor types [54, 55], and TERT overexpression in breast cancer has been correlated with poor response to chemotherapy [56, 57]. Whether *TERT* promoter mutations are prognostically significant in metaplastic carcinomas requires further study. Given that these mutations are considered to be among the earliest genetic events in tumorigenesis of other cancers [58, 59], it is interesting that only one of two metaplastic carcinomas with *TERT* promoter mutation in which both components were analyzed also harbored the mutation in paired high grade DCIS (Sp5), whereas intermediate grade DCIS associated with the other tumor (Sp1) lacked the mutation.

The absence of *TP53* mutations in spindle cell carcinomas in our study contrasts with the high frequency in other subtypes and in metaplastic carcinomas in general [24, 26]. Few studies have specifically analyzed the genetics of pure spindle cell metaplastic carcinomas, but both sequenced tumors reported by Ross et al also lacked *TP53* mutations [26]. Ng et al. reported *TP53* mutations in five of ten tumors in which a spindle cell component was sequenced, but these tumors were annotated to comprise other areas with non-spindle cell elements, including squamous, chondroid, osseous, and epithelioid areas, and so likely represent a heterogeneous group of mixed tumors [26]. Although we cannot definitely exclude small sample size bias in our study and in others [24, 26], it appears that *TP53* mutations are at least decreased in frequency if not absent from this subgroup, suggesting distinct pathogenesis from other metaplastic carcinomas. Two of the five tumors in our study were of intermediate nuclear grade; whether this is may be related to lack of *TP53* mutations is uncertain. Given that the distinction of metaplastic spindle cell carcinoma from phyllodes tumor with stromal overgrowth is a commonly encountered diagnostic dilemma, our results posit the testable hypothesis that *TP53* mutation testing or p53 immunostaining may be useful in this differential diagnosis, as malignant phyllodes tumors frequently harbor *TP53* mutations [33, 60]. On the other hand, we show that metaplastic carcinomas, like malignant phyllodes tumors, often harbor PI-3 kinase, Ras-Map kinase, and *TERT* promoter mutations, which are therefore not diagnostically useful in the differential [33, 60].

The repertoire of somatic mutations in DCIS associated with metaplastic carcinoma and its relationship to the invasive tumor has not been investigated prior to this study. We confirm the monoclonality of DCIS and paired metaplastic carcinoma. The presence of DCIS in association with a squamous, spindle cell, or otherwise mesenchymal-like tumor in the breast is often used as evidence to support a diagnosis of metaplastic carcinoma, and our genetic findings confirm the utility of this approach, regardless of the type of differentiation of the invasive or in situ tumor. Indeed, we show that even a histologically disparate DCIS with apocrine differentiation can be a direct precursor for spindle cell carcinoma.

Studies of conventional DCIS have demonstrated largely similar mutational and copy number profiles between the in situ and invasive components, albeit with increased numbers of copy number alterations in invasive cancer, and our results in metaplastic carcinomas are consistent with this [61–65]. As in conventional DCIS [62, 63, 65, 66], we show that *PIK3CA* alterations are early events in metaplastic carcinoma, with mutations in this and other growth-promoting PI-3 kinase/Ras-Map kinase pathway genes being present in clonal frequencies of associated DCIS and paired invasive tumors. In contrast, although p53 and Rb inactivation may also be early events in conventional [62, 64, 67–70] and metaplastic carcinoma-associated DCIS, mutations in *TP53*, *RB1* and *CDKN2A* were specifically associated with stromal invasion in individual metaplastic carcinomas. These findings support a convergent model of

tumor invasion, in which multiple pathways that are specific for individual tumors can be responsible for progression to invasion. A similar model has been proposed for conventional in situ-invasive ductal carcinoma [63, 71]. Our findings thus further support the idea that paired tumor analysis is most useful to uncover putative genetic drivers of invasion and help explain why studies comparing non-matched components have not consistently identified such factors [71–73]. These results also highlight the presence of intratumoral heterogeneity early during tumor development, with *TP53* subclones, as well as chromosomal gains and losses, present in DCIS but not invasive components of some tumors. We speculate that this likely reflects a bottleneck effect of tumor evolution in selection for invasive clones, as has previously been proposed for conventional ductal carcinomas [63, 71, 74]. Lastly, analysis of paired DCIS, invasive ductal carcinoma, and invasive squamous cell carcinoma in the same tumor (Sq4) highlights the stepwise dysregulation of oncogenic drivers such as *TP53*, *SMAD4*, and *KMT2D* during tumor evolution. The findings support a model in which heterologous components are directly evolved and genetically progressed from ductal carcinoma. Our study thereby confirms previous speculation based on a detailed histologic review of metaplastic carcinomas and further supports a similar conclusion of metaplastic carcinogenesis based on recent genetic analysis [75, 76].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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