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Guerini-Rocco, Elena; Hodi, Zsolt; Piscuoglio, Salvatore; Ng, Charlotte Ky; Rakha, Emad A; Schultheis, Anne M; Marchiò, Caterina; da Cruz Paula, Arnaud; De Filippo, Maria R; Martelotto, Luciano G; De Mattos-Arruda, Leticia; Edelweiss, Marcia; Jungbluth, Achim A; Fusco, Nicola; Norton, Larry; Weigelt, Britta; Ellis, Ian O; Reis-Filho, Jorge S. The repertoire of somatic genetic alterations of acinic cell carcinomas of the breast: an exploratory, hypothesis-generating study. JOURNAL OF PATHOLOGY. None pp: n/a-n/a.
DOI: 10.1002/path.4566

The repertoire of somatic genetic alterations of acinic cell carcinomas of the breast: an exploratory, hypothesis-generating study

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Conflicts of interest: The authors have no conflicts of interest to declare.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/path.4566

ABSTRACT

Acinic cell carcinoma (ACC) of the breast is a rare form of triple-negative (that is, estrogen receptor-negative, progesterone receptor-negative, HER2-negative) salivary gland-type tumor displaying serous acinar differentiation. Despite its triple-negative phenotype, breast ACCs are reported to have an indolent clinical behavior. Here, we sought to define whether ACCs have a mutational repertoire distinct from that of other triple-negative breast cancers (TNBCs). DNA was extracted from microdissected formalin-fixed paraffin-embedded sections of tumor and normal tissue from two pure and six mixed breast ACCs. Each tumor component of the mixed cases was microdissected separately. Tumor and normal samples were subjected to targeted capture massively parallel sequencing targeting all exons of 254 genes, including genes most frequently mutated in breast cancer and related to DNA repair. Selected somatic mutations were validated by targeted amplicon resequencing and Sanger sequencing. Akin to other forms of TNBC, the most frequently mutated gene found in breast ACCs was *TP53* (one pure and six mixed cases). Additional somatic mutations affecting breast cancer-related genes found in ACCs included *PIK3CA*, *MTOR*, *CTNNB1*, *BRCA1*, *ERBB4*, *ERBB3, INPP4B* and *FGFR2*. Copy number alteration analysis revealed complex patterns of gains and losses similar to those of TNBCs. Of the mixed cases analyzed, identical somatic mutations were found in the acinic and the high-grade non-acinic components in two out of four cases analyzed, providing evidence of their clonal relatedness. In conclusion, breast ACCs display the hallmark somatic genetic alterations found in high-grade forms of TNBC, including complex patterns of gene copy number alterations and recurrent *TP53* mutations. Furthermore, we provide circumstantial genetic evidence to suggest that ACCs may constitute the substrate for the development of more aggressive forms of triple-negative disease.

Keywords: massively parallel sequencing, immunohistochemistry, triple-negative, breast cancer, TP53

INTRODUCTION

Acinic cell carcinoma (ACC) is a vanishingly rare special histologic type of breast cancer, included in the spectrum of salivary gland-type tumors of the breast. This group comprises benign and malignant entities that bear histologic resemblance to their salivary gland counterparts [1]. ACC of the breast is defined as a malignant neoplasm similar to the ACCs of the parotid, showing serous acinar differentiation with zymogen-type cytoplasmic granules [2]. Morphologically, breast ACCs are characterized by infiltrative microglandular or solid-nest structures composed of cells with round-to-ovoid nuclei, discrete nucleoli and abundant eosinophilic-to-amphophilic cytoplasm containing small or coarse Paneth cell-like granules. Areas composed of clear cells with hypernephroid appearance can be present [3]. Immunohistochemical analyses of these cancers have revealed expression of S100-protein, epithelial membrane antigen (EMA) and serous differentiation markers, including amylase, lysozyme and alpha 1-antichymotrypsin [4]. Like many salivary gland-type tumors of the breast, ACCs display a triple-negative phenotype (that is, estrogen receptor (ER)-negative, progesterone receptor (PR)-negative and HER2-negative) [3].

Since its first description in 1996 [5], only thirty-nine cases of ACCs have been described in the breast, usually as case reports or small case series (reviewed in [4]). Distant recurrences have been described, but these are rare and usually related to the presence of a poorly differentiated invasive component [6-8]. Therefore, in contrast to the majority of triple-negative breast cancers (TNBCs) that show an overall aggressive clinical behavior, breast ACC is considered to be a favorable prognosis tumor [1,4,9].

There is burgeoning evidence to demonstrate that TNBC is an operational term that encompasses a heterogeneous collection of entities with distinct clinical behaviors and pathologic characteristics [10,11]. As a group, the majority of TNBCs are high-grade invasive ductal carcinomas of no special type, which display an aggressive clinical behavior, high response rates to conventional chemotherapy regimens and high levels of genetic instability

[10-13]. Massively parallel sequencing studies have revealed that, as a group, high-grade TNBCs are characterized by high numbers of somatic mutations, with *TP53* (82%) and *PIK3CA* (10%) being the most frequently mutated genes; however, a great deal of heterogeneity has been documented, with some tumors displaying over 200 somatic mutations affecting protein coding genes, whereas others display one or two somatic mutations [12,13].

Despite their triple-negative phenotype, salivary gland-type tumors of the breast often have genomic features that are distinct from those of high-grade TNBCs [10,14-17]. Secretory carcinoma and adenoid cystic carcinoma of the breast, as their salivary gland counterparts, are characterized by recurrent chromosomal translocations that result in specific fusion genes (*ETV6-NTRK3* and *MYB-NFIB*, respectively) [18-21]. Furthermore, breast adenoid cystic carcinomas display low levels of chromosomal instability and fewer copy number alterations than high-grade TNBCs, and patterns of somatic genetic alterations distinct from those of low-grade invasive ductal carcinomas [14,20,22]. A recent case report demonstrated that breast ACCs may develop in the context of *BRCA1* germline mutation carriers; genetic analysis of one such case revealed loss of heterozygosity (LOH) of the *BRCA1* locus and the presence of a *TP53* somatic mutation, akin to other types of *BRCA1*-related breast cancers [23]. Our group has recently demonstrated by Sanger sequencing analysis that, like common types of TNBC, *TP53* mutations are found in up to 80% of breast ACCs [24].

Here we sought to define whether breast ACCs are underpinned by somatic genetic alterations affecting the genes most frequently mutated in breast cancer and DNA repair related genes, as these tumors may arise in the context of *BRCA1* germline mutations. Given that a subset of ACCs of the breast are found in association with high-grade TNBCs, we investigated whether ACCs might constitute the substrate for the development of a more aggressive form of triple-negative disease.

MATERIALS AND METHODS

Cases and Immunohistochemistry

Seven cases with a diagnosis of ACC of the breast were obtained from the consultation files of one of the authors (IOE) and one case from the pathology archives of Vall d'Hebron University Hospital, Barcelona, Spain. Histologic and immunohistochemical details of the cases included in this study are reported elsewhere [24]. All cases were reviewed by four of the authors (ZH, EAR, IOE and JSR-F), and classified as pure ACCs (n=2) and mixed ACCs (n=6; Table 1). The samples were anonymized prior to analysis. This study was approved by the local ethics committees from the contributing institutions. Patient consents were obtained if required by the protocols approved. Hematoxylin and eosin-stained sections of each case were further reviewed by four pathologists (EGR, AMS, CM and JSR-F), who analyzed the morphologic features of each component in detail following previously described criteria [3]. In addition, the presence of invasive components of different histologic types and their histologic characteristics were assessed. Each tumor component was graded separately according to the Nottingham grading system[25]. Immunohistochemical analysis was performed on representative formalin-fixed paraffin embedded (FFPE) sections of each sample using validated antibodies against ER, PR, HER2, lysozyme, Ki67 and p53. Details and scoring systems are available in the Supplementary Methods.

Microdissection and DNA extraction

Eight-µm-thick sections of representative FFPE blocks of the tumor and normal breast tissue from each case were stained with nuclear fast red and microdissected using a sterile needle under a stereomicroscope (Olympus SZ61) to ensure >80% of tumor cell content and that the normal tissue was devoid of any neoplastic cells as previously described [26,27]. Morphologically distinct components of each mixed ACC (i.e. acinic and non-acinic components) were microdissected separately [27]. Genomic DNA was extracted from each

tumor component and matched normal tissue using the DNeasy Blood and Tissue Kit (Qiagen) and quantified using the Qubit Fluorometer assay (Life Technologies) as previously described[28].

Targeted capture massively parallel sequencing

Sufficient DNA was obtained from normal and tumor samples from two pure ACCs, six acinic components of mixed cases, from which DNA could also be obtained from the non-acinic components in four cases. Normal and tumor DNA samples were subjected to targeted capture massively parallel sequencing at the Integrated Genomics Operation (IGO), Memorial Sloan Kettering Cancer Center, using a customized breast cancer panel targeting all exons of 254 genes recurrently mutated in breast cancer and DNA repair-related genes (Supplementary Table S1) using custom oligonucleotides (NimblegenSeqCap) as previously described [26,29]. Massively parallel sequencing reads were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner [30]. Local realignment, duplicate removal and quality score recalibration were performed using the Genome Analysis Toolkit [31]. Somatic single nucleotide variants (SNVs) were identified using MuTect [32]; small insertions and deletions (indels) were identified using Strelka and VarScan 2 [33,34] and further curated by manual inspection. SNVs and indels with mutant allelic fraction of <1% and/or supported by <5 reads were disregarded [35]. All mutations identified by targeted capture sequencing were subsequently subjected to validation using a targeted amplicon sequencing approach (Supplementary Methods, Supplementary Table S2). In addition, selected mutations were validated by Sanger sequencing as previously described [28] (Supplementary Methods, Supplementary Table S3). ExomeCNV was employed to determine whether a gene harboring a somatic mutation was also targeted by LOH [36]; the loci of mutated genes were also manually curated to resolve potential LOH through the analysis of chromosomal copy number plots generated by VarScan 2 [33]. Copy number variations were analyzed using VarScan 2 [33] and segmentation was performed as previously described (Supplementary Methods) [37]. Targeted capture massively parallel

sequencing data have been deposited in the Sequence Read Archive (SRA) under accession number SRP052551.

The potential functional effect of each SNV was investigated using a combination of MutationTaster [38] and CHASM [39]. The mutations identified as non-deleterious/passengers by both computational prediction algorithms were considered passenger alterations [40]. Genes affected by non-passenger mutations were further annotated according to their presence in three cancer gene datasets, Kandoth *et al*. [41], the Cancer Gene Census [42] and Lawrence *et al*. [43]. Cancer cell fractions were defined using ABSOLUTE [44] and clonal composition analysis was carried out using SubcloneSeeker [45] (Supplementary Methods).

RESULTS

ACCs are low-grade TNBCs

The pure ACCs and the ACC components of all mixed cases included in this study displayed the cardinal histologic features of breast ACCs, including infiltrating microglandular growth pattern and the presence of cytoplasmic secretory granules (Figures 1 and 2, Table 2). The two pure ACCs were of grades 1 and 2. The ACC components of the mixed cases were of grade 1 and 2 in four and three cases, respectively (Table 2), whereas the non-acinic components of mixed tumors included five grade 3 invasive ductal carcinomas of no special type (IDC-NST), one grade 2 IDC-NST and one grade 3 metaplastic carcinoma (Figure 2, Table 2). Immunohistochemical analysis revealed that pure ACCs and the ACC components of the mixed cases displayed the characteristic triple-negative phenotype and immunoreactivity for lysozyme, a serous differentiation marker (Figures 1 and 2, Table 2). All non-ACC components tested showed a triple-negative immunohistochemical profile (Table 2).

ACCs display a complex repertoire of mutations with recurrent *TP53* somatic mutations

Targeted capture massively parallel sequencing analysis yielded a median depth of coverage of 107x to 155x, 157x to 653x and 130x to 818x in pure ACCs (n=2), acinic components of mixed cases (n=6), and non-acinic components of mixed cases (n=4, Table 2), respectively. Targeted amplicon resequencing analysis validated 133 of the 134 single nucleotide variants and indels identified at a median depth of coverage of 7,361x, 6,044x to 7,745x and 6,876x to 7,448x in pure ACC (n=1), acinic components of mixed cases (n=6) and non-acinic components of mixed cases (n=4, Table 2), respectively (Supplementary Table S2). The two pure and six acinic components of mixed carcinomas displayed a median of 5 somatic mutations affecting the 254 genes tested (range 2 to 11, Table 2 and Supplementary Table S4). A re-analysis of the 78 TNBCs from The Cancer Genome Atlas (TCGA) breast cancer study revealed a median frequency of 3 somatic mutations (range 0 to 13) affecting the exons of the 254 genes included in our targeted panel [12], suggesting that there was a higher number of mutations affecting these 254 genes in ACCs than in the TNBCs analyzed by TCGA (Wilcoxon test, p=0.003). Although these differences may reflect the biology of ACCs, it is equally plausible that they may stem from the fact that in our study, cases were microdissected and sequencing depth was substantially higher than that of cases analyzed by TCGA.

TP53 was the most commonly mutated gene, being present in seven acinic cell components (one of two pure ACCs, and in six ACC components of mixed cases; Figure 3). It should be noted that the *TP53* wild-type ACCs displayed histologic features similar to the *TP53* mutant cases included in this study. Additional recurrently mutated genes included lysine (K)-specific methyltransferase 2D (*KMT2D,* also known as *MLL2*), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (*ERBB4*) and nebulin (*NEB*, Figure 3). Other non-synonymous somatic mutations affecting genes frequently mutated in breast cancer found in ACCs included phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

(*PIK3CA*), mechanistic target of rapamycin (*MTOR*), β-catenin (*CTNNB1*), breast cancer 1 early onset (*BRCA1*), v-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*), inositol polyphosphate-4-phosphatase type II (*INPP4B*) and fibroblast growth factor receptor 2 (*FGFR2*). The truncating *BRCA1* somatic mutation identified in case 17 was coupled with LOH of the wild-type allele (Figure 3) providing evidence of complete loss of *BRCA1*. Given this observation and the fact that an ACC has been reported in a patient with a *BRCA1* germline mutation [23], we investigated whether the patients included in this study harbored germline disease-causing mutations affecting DNA-repair related breast cancer susceptibility genes, including *BRCA1*, *BRCA2*, *PALB2*, *RAD51C*, *RAD51D*, *BRIP1* and *FAM175A*. This analysis revealed the presence of a germline frame-shift mutation of *BRCA1* (E23*) in case 10. In this case, somatic LOH of the *BRCA1* wild-type allele was observed (Supplementary Figure S1).

Sanger sequencing analysis of selected mutations confirmed the *TP53*, *PIK3CA, KMT2D*, *ERBB4* and *NEB* somatic mutations (Supplementary Figure S2). Furthermore, immunohistochemical analysis of p53 revealed strong nuclear expression in the cases harboring a *TP53* missense mutation; cases 15 and 17, which harbored *TP53* frameshift mutations coupled with LOH of the wild-type allele, lacked p53 expression, consistent with the type of *TP53* somatic genetic alteration found (Table 2).

In cases 14 and 16, the two morphologically distinct ACC components were successfully sequenced and were shown to harbor somatic mutations in common. In case 14, two identical somatic mutations were found in the grade 1 and grade 2 ACC components (i.e. *TP53* R273C and *INPP4B* Q306K); however, two and four somatic mutations were restricted to the grade 1 and grade 2 components, respectively (Figure 4). Interestingly, distinct somatic mutations targeting the cubulin (*CUBN*) gene were found in each component, suggestive of a potential convergent phenotype. In case 16, the microglandular and clear cell hypernephroid ACC components shared seven identical somatic mutations, including a

TP53 V157D and a hotspot *PIK3CA* E542K mutations (Figure 4); however, the microglandular ACC component harbored four additional somatic mutations not identified in the clear cell component, including an *ERBB3* M60R somatic mutation. Evidence of a potential convergent phenotype was also observed in this case, given that the microglandular component harbored a *CTNNB1* N387K mutation, whereas the clear cell component harbored a *CTNNB1* K335T mutation (Figure 4).

ACCs of the breast display complex patterns of gene copy number alterations

Consistent with the presence of *TP53* somatic mutations, gene copy number analysis revealed that ACCs displayed complex patterns of copy number alterations, with multiple gains and losses across the entire genome. Recurrent changes included gains of 1q, 2q, 8q and losses of 3p, 5q, 12q, 13q, 14q, 17p and 17q (Supplementary Figure S3). Furthermore, the ACC components of cases 7 and 10 displayed amplification of the 1q43 region involving the v-akt murine thymoma viral oncogene homolog 3 (*AKT3*) and formin 2 (*FMN2*) gene loci (Supplementary Figure S1 and Supplementary Table S5). The 8q24.12 locus was amplified in the ACC component of cases 9 and 14, encompassing the DEP domain containing MTOR-interacting protein (*DEPTOR*) gene locus, and the 8q24.3 locus was amplified in the ACC component of cases 9 and 17, encompassing the focal adhesion kinase, protein tyrosine kinase 2 (*PTK2*) gene (Figures 4 and 5, Supplementary Figure S1, Supplementary Table S5). Furthermore, we identified a homozygous deletion involving the AHNAK nucleoprotein 2 (*AHNAK2*) gene in the ACC of case 10 (Supplementary Figure S1, Supplementary Table S5).

Genomic profiles of acinic and non-acinic cell components of mixed ACCs

Massively parallel sequencing results were available for both the acinic and non-acinic components of four mixed cases, including three grade 3 invasive ductal carcinomas of no special type and one grade 3 metaplastic carcinoma (Table 2). The ACC and the high-grade TNBC components were found to be clonally related in two cases based on the mutational

and copy number profiling (cases 9 and 16, Figures 4, 5 and 6; Supplementary Tables S4 and S5). Additional somatic mutations were found in the high-grade triple-negative carcinoma component of case 9, including a clonal *PIK3CA* E542K hotspot mutation, followed by a subclonal frame-shift mutation affecting the *bona fide* cancer gene *KMT2D* and a subclonal missense mutation affecting the *AK9* gene (Figures 5 and 6). In case 16, somatic mutations affecting *ERBB3*, *NEB and CTNNB1* N387K were identified only in the ACC component. On close inspection, a progression from the classic ACC to the clear cell hypernephroid component to the metaplastic carcinoma could be observed in case 16 on the basis of the patterns of mutations and gene copy number alterations. A somatic *CTNNB1* K335T mutation and a focal amplification of 5p13.1-p12, encompassing the loci of the growth hormone receptor gene (*GHR*) and the rapamycin-insensitive companion of mTOR gene (*RICTOR)* were only present in the clear cell and in the metaplastic carcinoma components (Figures 4 and 6, Supplementary Table S5).

No shared somatic mutations were detected in the ACC and the high-grade triple-negative carcinoma components of cases 14 and 15 (Figures 4 and 5); the components of these cases also displayed distinct copy number profiles (Figures 4 and 5). The invasive component of case 14 harbored a 9p24.2-21.3 amplification and a homozygous deletion involving the retinoblastoma (*RB1*) gene locus (Figure 4 and Supplementary Table S5). In case 15, we identified a homozygous deletion in 14q21.2 encompassing the Fanconi anemia, complementation group M (*FANCM*) gene restricted to the invasive component (Figure 4 and Supplementary Table S5).

Taken together, our results provide circumstantial genetic evidence to suggest that, in some cases, ACCs may constitute the substrate for the development of invasive TNBCs of higher histologic grade or of more aggressive subtypes.

DISCUSSION

Here we demonstrate that breast ACCs harbor a high mutational burden, recurrent *TP53* mutations, *BRCA1* germline and somatic pathogenic mutations, and complex patterns of gene copy number alterations, in a way akin to high-grade TNBCs. Our findings corroborate the notion that TNBCs constitute a heterogeneous collection of diseases[10,11] with varying morphologic and molecular characteristics and clinical behaviors, whose unifying characteristics include the lack of ER, PR and HER2 expression and recurrent *TP53* somatic mutations [12,13]. The pattern of *TP53* mutations found in TNBCs differs from that observed in ER-positive tumors, with an enrichment for nonsense SNVs and indels [12,13]. The frequency of somatic *TP53* mutations found in breast ACCs is similar to that of high-grade TNBCs (87% vs 82%, respectively); in contrast, however, ACCs preferentially harbored missense mutations, including mutations affecting the R273 hotspot found in three ACCs (cases 7, 10 and 14). Additional mutations and amplifications found in breast ACCs involved genes or pathways that have been previously reported to be altered, although some at low frequency, in high-grade TNBCs, including *PIK3CA*, *FGFR2*, *INPP4B, ERBB4, AKT3, FMN2, DEPTOR* and *PTK2* according to a reanalysis of the breast TCGA data (www.cbioportal.org; accessed 01-15-2015) [12,46]. TNBCs often display high levels of genomic instability that result in multiple low-level gains and losses throughout the genome, with a few high-level amplifications [47]. Our results reveal a similar complexity in the pattern of gains and losses across the entire genome of the ACCs, including recurrent copy number alterations in regions often altered in other forms of triple-negative disease, such as 8q gain and 5q loss [12,47,48]. Finally, consistent with the notions that i) *BRCA1* germline mutations preferentially predispose to the development of breast cancers with a triple-negative phenotype [11] and ii) 11%-16% of TNBCs harbor *BRCA1* germline or somatic mutations [49-51], one of the pure ACCs analyzed displayed a *BRCA1* nonsense somatic mutation coupled with LOH of the *BRCA1* wild-type allele and a somatic *TP53* mutation, and another mixed ACC harbored a germline *BRCA1* mutation coupled with somatic LOH of the wild-type allele and a *TP53* somatic mutation. This finding suggests that the presence of *TP53* and

BRCA1 loss-of-function may not be sufficient for the development of high-grade TNBCs. In fact, conditional mouse models of *BRCA1* and *TP53* have been shown to result in the development of rather heterogeneous tumors; although the majority of lesions are of high histologic grade and display the cardinal features of human high-grade TNBCs, 4% to 31% of the mammary gland tumors those animals developed were of grades 1 or 2 [52,53]. Ingenuity Pathway Analysis employing genes mutated in pure and/ or mixed ACCs and in common forms of TNBCs from TCGA revealed a significant enrichment for genes related to cellular growth and proliferation and cell development in both datasets (score 37 and 38 respectively; Supplementary Figure S4). By contrast, a significant enrichment for genes related to DNA recombination and repair processes was only found in TNBCs (score 51, Supplementary Figure S4) but not in ACCs. Taken together, our findings support the contention that, despite its reported indolent clinical behavior, breast ACCs are closely related to the common forms of high-grade TNBC but display a relatively more limited genomic complexity. It is plausible that the differences in clinical behavior between ACCs and common forms of triple-negative disease stem from the lower proliferation rates, as defined by Ki67, and histologic grades found in ACCs than in common forms of TNBCs (Table 2).

The presence of poorly differentiated tumors adjacent to ACCs is not an uncommon finding in the breast [7,23] and it has also been reported in the context of ACCs of the salivary glands [54]. The non-ACC components of the mixed cases included in this study were all high-grade, highly proliferative triple-negative breast carcinomas (Figure 2 and Table 2). Our analyses revealed identical somatic mutations and similar patterns of copy number alterations in the ACC and the high-grade triple-negative carcinoma components of two of the four cases. In both cases, a higher degree of genomic complexity was observed in the high-grade lesion; in one of the cases, a clear stepwise increase in the genomic complexity from the microglandular ACC component to the clear cell ACC component to the metaplastic carcinoma component was observed (Figure 4). In this case, whilst the microglandular ACC

component displayed a *CTNNB1* N387K mutation, the clear cell ACC and the metaplastic carcinoma components harbored a *CTNNB1* K335T mutation, suggesting a convergent phenotype and a potential progression mechanism. These findings provide direct evidence that in some cases, classic ACCs are an indolent form of triple-negative disease that may constitute the substrate for the development of high-grade TNBCs.

In the two cases of ACCs associated with high-grade TNBCs that displayed distinct constellations of somatic mutations and gene copy number alterations (cases 14 and 15; Figures 4 and 5), *TP53* mutations were found to be restricted to the ACC component and massively parallel sequencing and Sanger sequencing failed to identify *TP53* mutations in the high-grade triple-negative carcinoma components of these cases. Potential alternative driver genetic alterations found in these components included a RAD50 homolog (*RAD50*) gene S560R mutation coupled with LOH of the wild-type allele in case 14, and a core-binding factor, beta subunit (*CBFB*) gene truncating mutation in case 15 (Figures 4 and 5). Although no somatic mutations and gene copy number alterations were found in common between the acinic and high-grade triple-negative components of these cases, a finding that could be interpreted as suggestive of a non-clonal origin of these lesions, we cannot rule out a potential common origin on the basis of genetic alterations not surveyed in this study.

This study has several limitations. First, given the rarity of breast ACCs, the sample size of our study is relatively small, in particular of pure ACCs (n=2). It should be noted, however, that this study represents the largest cohort of breast ACCs subjected to massively parallel sequencing analysis to date. Second, given that all cases were formalin-fixed, paraffin-embedded and that the lesions were rather small, we were only able to subject these samples to targeted capture massively parallel sequencing. Therefore, we cannot rule out the presence of a pathognomonic mutation or fusion gene affecting a gene not included in this study that might define ACCs of the breast. This is, however, unlikely, given that breast and salivary gland tumors driven by highly recurrent pathognomonic fusion genes or

mutations often lack *TP53* mutations and have simple patterns of gene copy number alterations (e.g. adenoid cystic carcinomas of the breast[20] and polymorphous low-grade adenocarcinomas [28]). Our findings, however, warrant further investigation of the repertoire of somatic mutations and expressed fusion genes in breast ACCs. Finally, given the retrospective nature of this study and the fact that most samples were obtained from the consultation files of one of the authors, we were unable to perform a detailed survival analysis.

In conclusion, ACCs of the breast are part of the spectrum of TNBCs and despite their low-grade and reported indolent clinical behavior, these tumors display the cardinal genomic features documented in high-grade forms of triple-negative disease. We have also provided circumstantial data to suggest that, the ACC may constitute the substrate for the development of a high-grade TNBC in a subset of cases, given the presence of identical truncal/ clonal mutations present in both the ACC and the high-grade TNBC. Finally, our observations reiterate the fact that TNBC is a mere operational term [11] and that histopathologic analysis provides important information about the biology and clinical behavior of TNBCs.

ACKNOWLEDGMENTS

SP is funded by a Susan G Komen Postdoctoral Fellowship Grant (PDF14298348), AMS by a stipend from the German Cancer Aid (Dr. Mildred Scheel Stiftung), and CM by AIRC (MFAG13310).

AUTHORS CONTRIBUTIONS

JSR-F, BW and IOE conceived and supervised the study. IOE, ZH, EAR and LDM-A provided the samples; EGR, AMS, CM, NF, ME, ZH, EAR, IOE and JSR-F performed the histological review. EGR, AMS, CM and NF performed the sample microdissection; EGR, SP, ACP, MRF, LGM, AAJ and CKYN carried out experiments and analyzed data; EGR, SP,

CKYN, BW and JSR-F interpreted results and drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

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TABLES

Table 1: Clinico-pathologic features of the acinic cell carcinomas included in this study.

Case	Age at diagnosis (years)	Tumor size (mm)	Node status	Follow-up (months)	Follow-up (status)	Diagnosis
7	36	50	10/17	24	DOD	mixed IDC-NST and ACC
9	55	19	0/8	132	DF	mixed IDC-NST and ACC
10	34	NA	NA	NA	NA	mixed IDC-NST and ACC
12	42	NA	NA	NA	NA	pure ACC
14	34	36	0/3	15	DF	mixed IDC-NST and ACC
15	48	20	NA	60	DF	mixed IDC-NST and ACC
16	70	NA	NA	NA	DR	mixed metaplastic carcinoma and ACC
17	35	NA	2/22	72	DR	pure ACC

ACC, acinic cell carcinoma; DOD, dead of disease; DF, disease free; DR, disease recurrence; IDC-NST, invasive ductal carcinoma of no special type; NA, not available.

Table 2: Histologic and immunohistochemical features of the acinic cell carcinomas included in this study.

Case	Pure vs mixed	Growth pattern	Cytoplasmic granules	Grade	TNP	LYS	*TP53* mutation, p53 IHC	Ki67 *	Sequence depth (capture and amplicon sequencing)	Somatic mutations (n)	Non-acinic component	Grade	TNP	LYS	*TP53* mutation, p53 IHC	Ki67 *	Sequence depth (capture and amplicon sequencing)	Somatic mutations (n)
Pure ACCs																		
12	pure	Microglandul	small	2	Y	+	WT, -	21 %	107x	9								
17	pure	Microglandul	small and	1	Y	+	P250fs,	15 %	155x and	8								
Mixed ACCs																		
7	mixed	Clear cell	absent	1	Y	+	R273C,	25 %	157x and	2	IDC-NST	2	Y	+	NP, +	NP	NP	NP
9	mixed	Microglandul	small and	1	Y	+	M237I, +	12 %	653x and	4	IDC-NST	3	Y	+	M237I,+	53 %	231x and	7
10	mixed	Microglandul	small	1	Y	+	R273H,	22 %	339x and	5	IDC-NST	3	Y	+	NP, +	NP	NP	NP
14	mixed	Microglandul	small and	1	Y	+	R273C,	14 %	357x and	4	IDC-NST	3	Y	-	WT, -	92 %	818x and 7,182x	12
	mixed	Microglandul	small and	2			R273C,	27 %	478x and	6								
15	mixed	Microglandul	small	1	Y	+	S303fs,	12 %	285x and	4	IDC-NST	3	Y	-	WT, -	33 %	237x and	3
16	mixed	Microglandul	small	1	Y	+	V157D,	11 %	218x and	11	MBC	3	Y	-	V157D, +	44 %	130x and 7,448x	7
	mixed	Clear cell	absent	2			V157D, +	19 %	358x and 7,200x	8								

IDC-NST, invasive ductal carcinoma of no special type; LYS, lysozyme; MBC, metaplastic breast carcinoma; NP, not performed; TNP, triple-negative phenotype (i.e. estrogen receptor, progesterone receptor and HER2-negative); WT, wild-type; Y, yes; +, positive; -, negative; *, hotspot areas were selected.

FIGURE LEGENDS

Figure 1

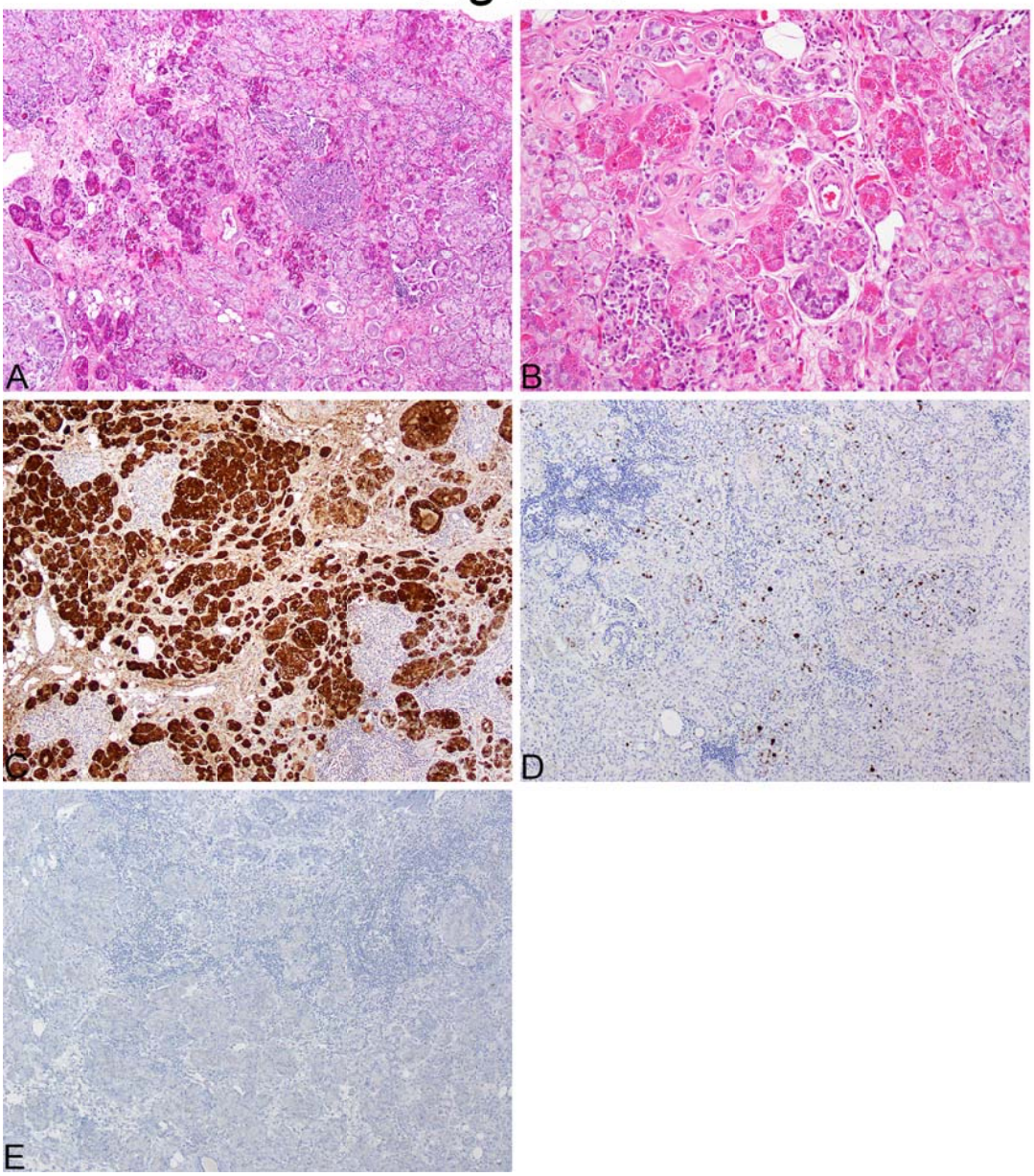

Figure 1: Pure acinic cell carcinoma of the breast.

Representative micrographs of a pure acinic cell carcinoma (Case 17, A, B) displaying lysozyme (C) and intermediate Ki67 expression (D). Note that this case, which harbored a *TP53* somatic frameshift mutation and LOH of the wild-type allele, lacked p53 expression (E). Original magnification x40, A, B, C, D and E.

Figure 2

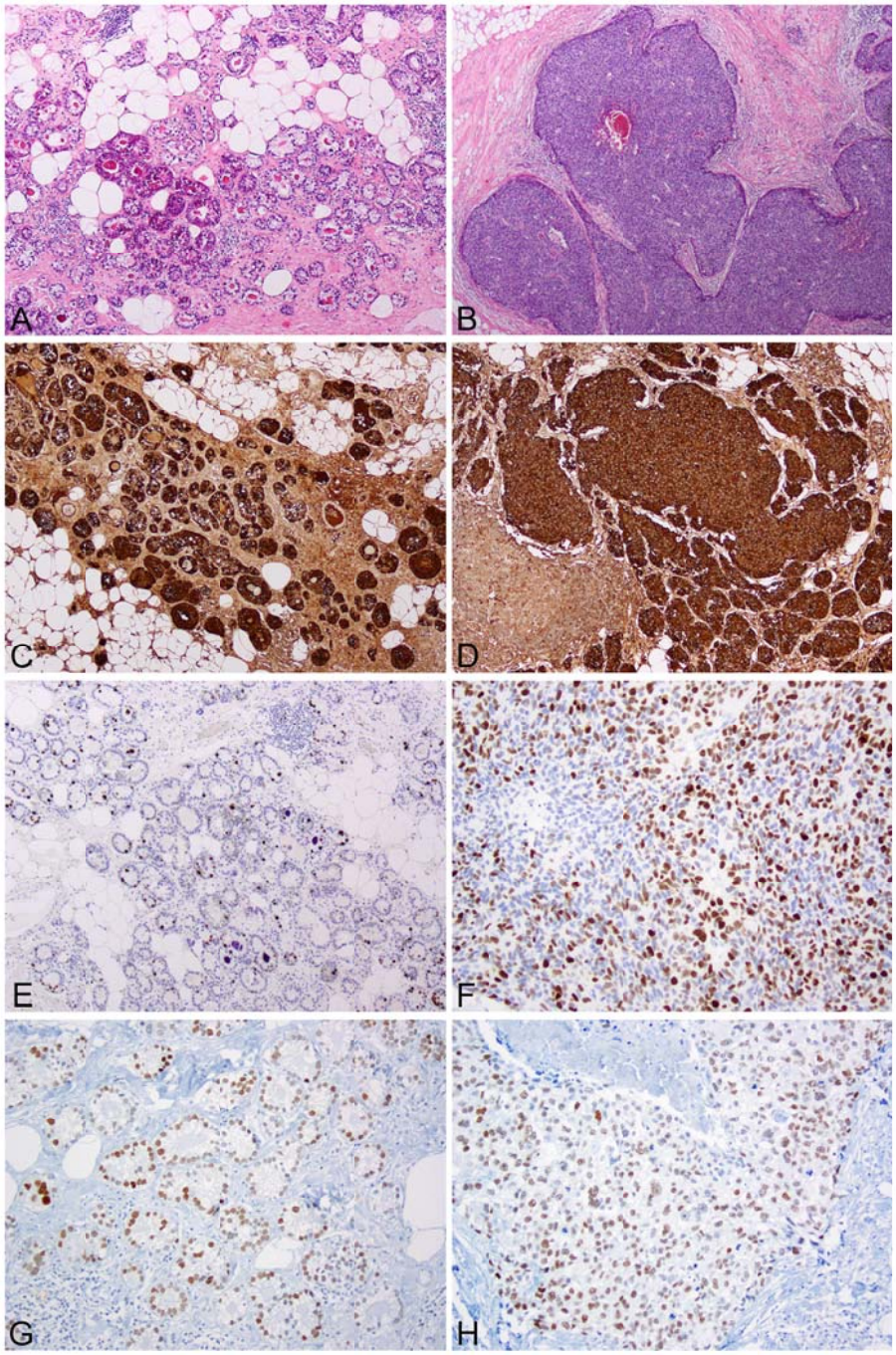

Figure 2: Mixed acinic cell carcinoma of the breast.

Representative micrographs of the acinic (A) and high-grade triple-negative invasive ductal carcinoma of no special type (B) components of a mixed acinic cell carcinoma (Case 9), where both components expressed lysozyme (C, D), Ki67 (E, F) and p53 (G, H). Note the substantially higher Ki67 labeling index in the high-grade triple-negative invasive ductal

carcinoma of no special type component (F) than in the acinic cell carcinoma component (E). Original magnification x40, A, B, C, D and E; x100, F, G and H.

Figure 3

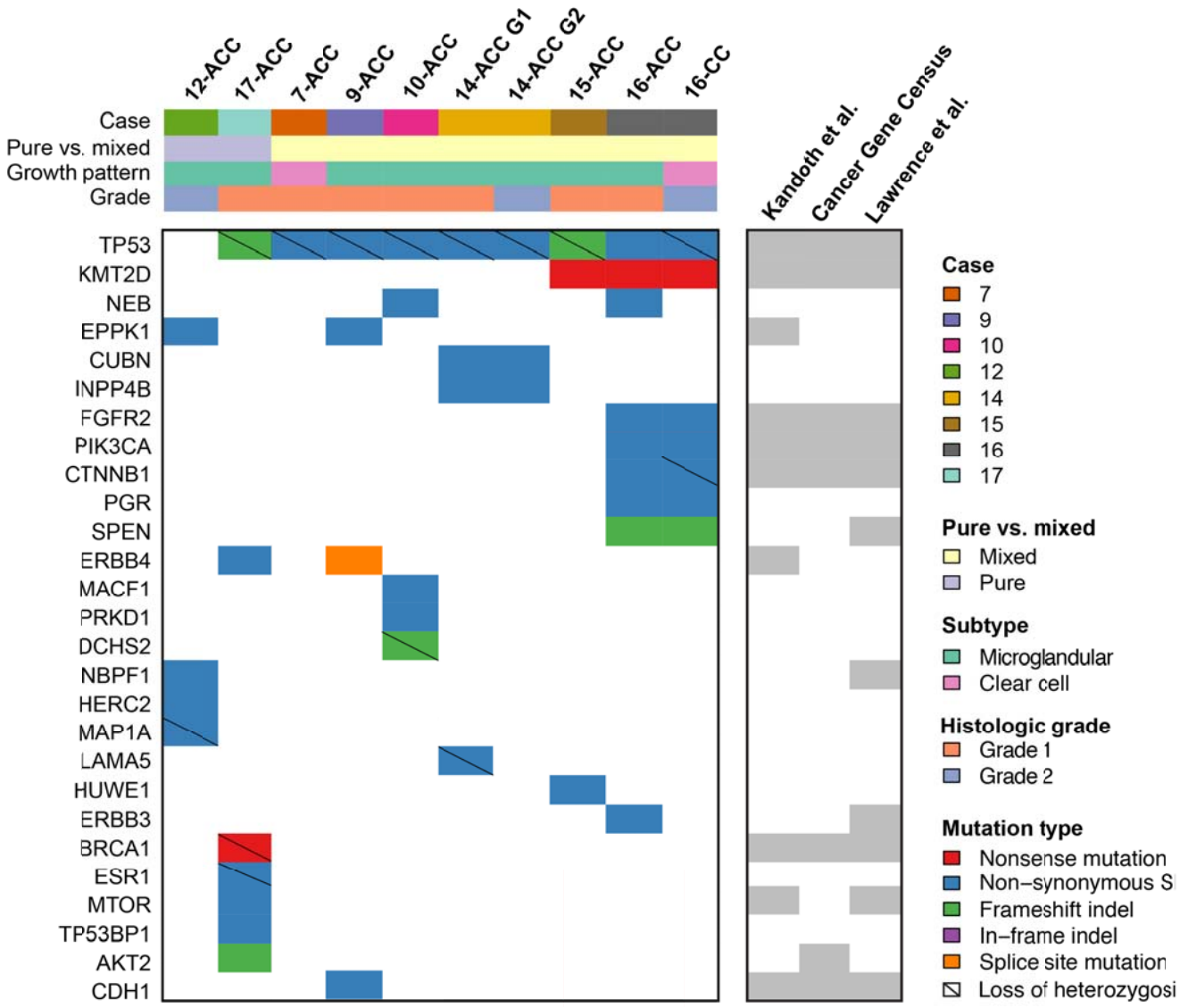

Figure 3: Non-synonymous somatic mutations detected by targeted capture massively parallel sequencing in acinic cell carcinomas of the breast.

Heatmap indicating the non-synonymous somatic mutations considered not to be neutral by either MutationTaster or CHASM. Cases are represented in columns; genes are depicted in rows. Mutation types are color-coded according to the legend. The presence of loss of heterozygosity of the wild-type allele in association with the somatic mutation is depicted by

a diagonal bar. On the right, the membership of each gene in three cancer gene datasets, Kandoth *et al*. [41], Cancer Gene Census [42] and Lawrence *et al*. [43], is reported. ACC, acinic cell carcinoma; CC, clear cell focus.

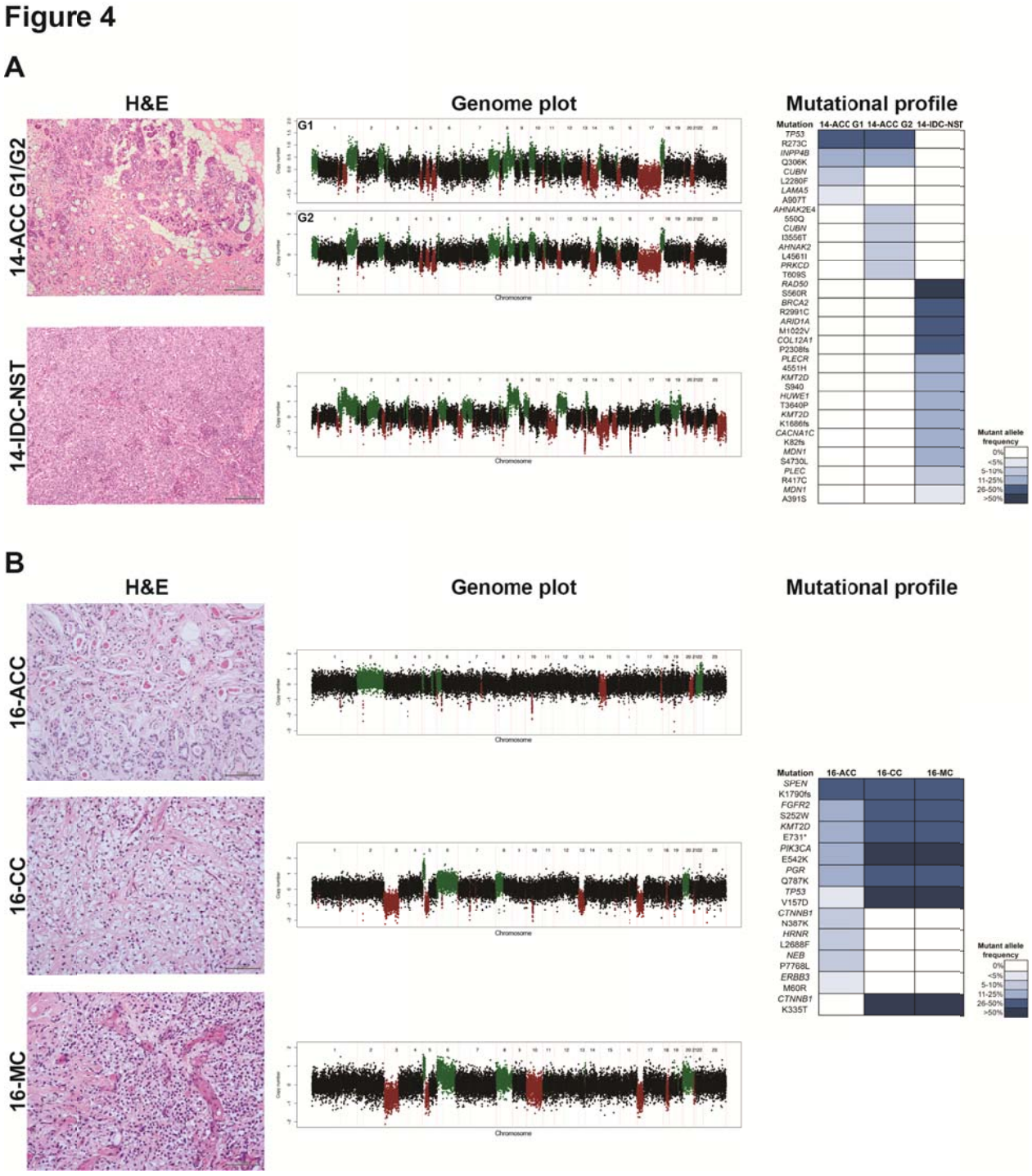

Figure 4: Mixed acinic cell carcinomas of the breast displaying morphologic and genetic heterogeneity.

Each panel depicts representative micrographs of each component, its respective genome plot and repertoire of non-synonymous somatic mutations. In the genome plots, smoothed Log_2 ratios were plotted on the y-axis according to their genomic positions indicated on the x-axis. Gains and losses are plotted in green and red, respectively. On the right, diagrams depicting the mutations identified in each component of each case. In these diagrams, each mutation is color-coded on the basis of its mutant allele fraction, as explained in the color key. A, case 14; B, case 16. Scale bars: 500μm.

ACC: acinic cell carcinoma; CC: clear cell ACC component; G1, grade 1; G2, grade 2; IDC-NST: invasive ductal carcinoma of no special type; MC: metaplastic carcinoma.

Figure 5

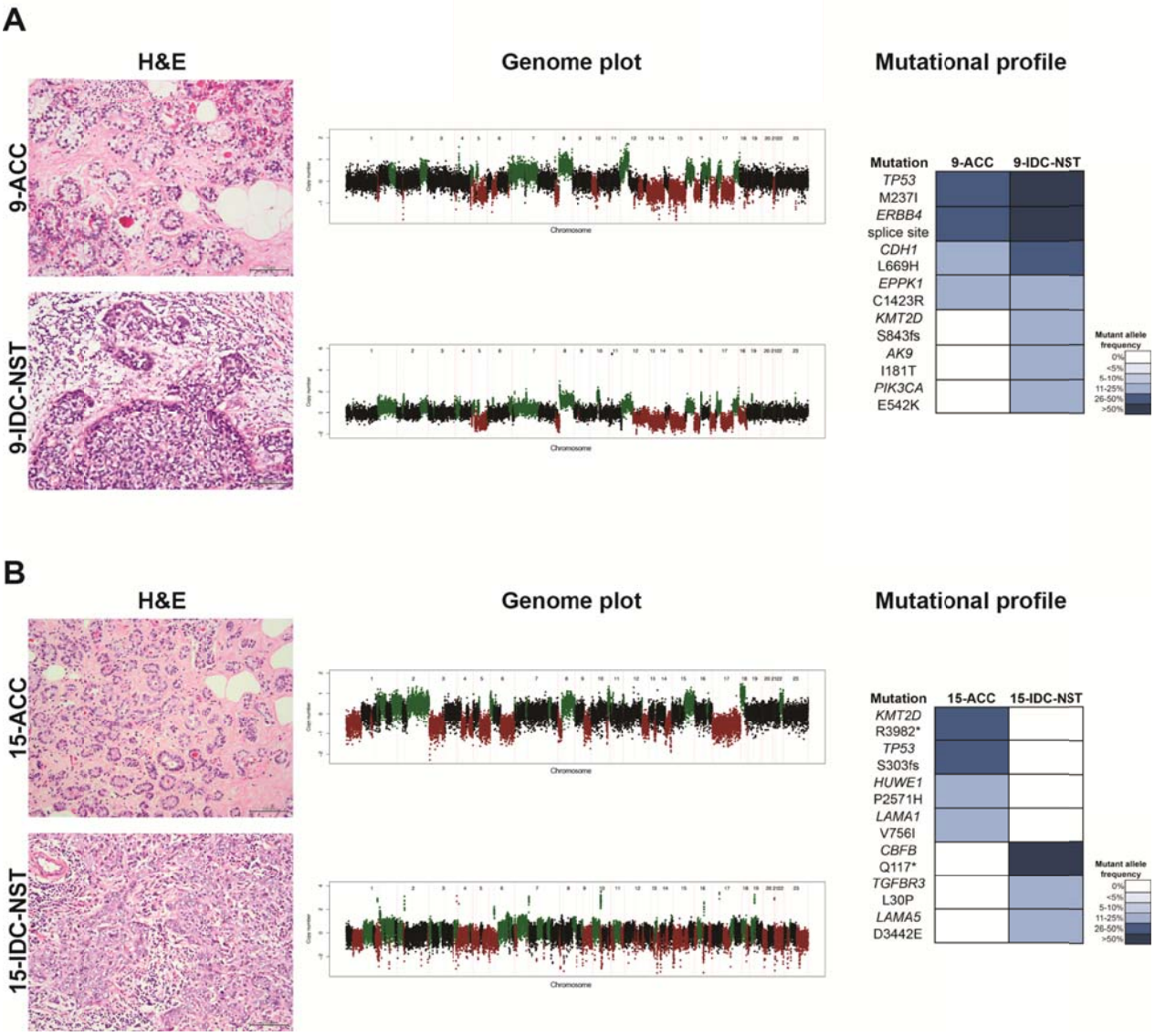

Figure 5: Genomic profiling of mixed acinic cell carcinomas of the breast.

Each panel depicts representative micrographs of each component, its respective genome plot and repertoire of non-synonymous somatic mutations. In the genome plots, smoothed Log_2 ratios were plotted on the y-axis according to their genomic positions indicated on the x-axis. Gains and losses are plotted in green and red, respectively. On the right, diagrams depicting the mutations identified in each component of each case. In these diagrams, each mutation is color-coded on the basis of its mutant allele fraction, as explained in the color

key. A, case 9; B, case 15. Scale bars: 500μm. ACC: acinic cell carcinoma; IDC-NST: invasive ductal carcinoma of no special type.

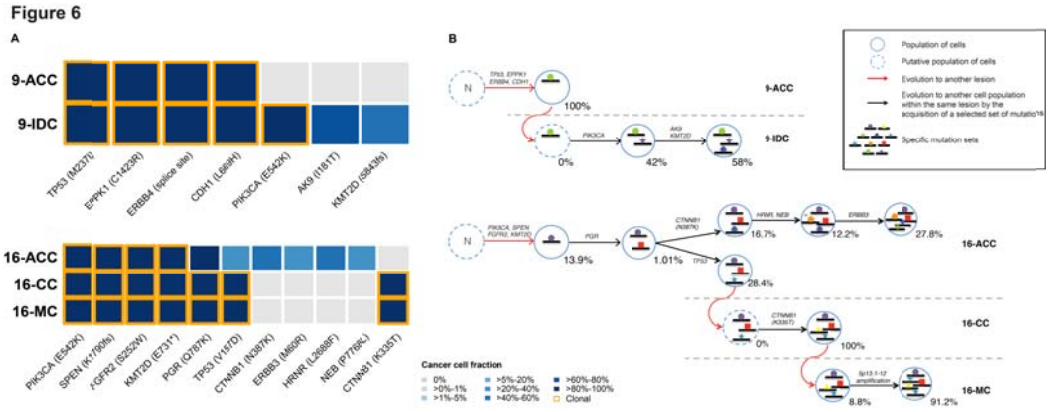

Figure 6: Cancer cell fractions and clonal composition of histologically distinct components of clonally-related mixed acinic cell carcinomas. (A) Representative cancer cell fraction (i.e. estimated percentage of cancer cells harboring a given validated somatic mutation) as defined by ABSOLUTE [44] through the integration of tumor cellularity, ploidy, gene copy number and mutant allele fractions in each histologically-distinct component of mixed acinic cell carcinomas where the acinic and non-acinic components were found to be clonally-related. (B) Putative evolution of clones in each lesion, where each circle represents a (sub)clone with a specific subset of genetic alterations. Each black arrow represents the acquisition of somatic genetic alterations, listed above or below it, which define the emergence of the respective clone. Red solid arrows depict the divergence of a cell population from one lesion to another. Decomposition of genetically distinct clones and clonal evolution in lesions from case 9 and case 16 were performed using the results from ABSOLUTE[44] and SubcloneSeeker [45]. The percentages indicate the prevalence of each clone in each morphologically distinct component of each case. ACC, acinic cell carcinoma; CC, clear cell acinic cell carcinoma; IDC, invasive ductal carcinoma of no special type; MC, metaplastic carcinoma.