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Original contribution

Revisiting multifocal breast cancer: a clonality study of ductal carcinoma using whole exome sequencing☆

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Summary Multifocal breast cancer (MFBC), ductal type, has been hypothesized to arise by one of two mechanisms: either through intramammary/intralymphatic spread from a single index tumor (MBC-1), or as multiple independent tumors with each focus carrying its corresponding ductal carcinoma in-situ (MBC-2). In order to improve our understanding of MFBC pathogenesis, we employed laser capture microdissection coupled with whole-exome sequencing to study clonal origin in MFBC. We selected three cases of MBC-1 (C1 to C3) and MBC-2 (C4 to C6) and analyzed three foci from each case. MBC-1 cases were histologically similar and showed a strong predilection for satellite foci, vascular invasion and nodal metastasis when compared to MBC-2. Our bioinformatics approach provided strong evidence for clonal relationships in MBC-1, as demonstrated by distinct clusters of genes conserved across all tumor foci. Conversely, no gene clusters were shared across all the foci in MBC-2, suggesting multiple independent tumors. These findings provide further support for the two distinct pathogenetic mechanisms in MFBC.

1. Introduction

Multiple ipsilateral invasive ductal carcinomas of the breast remain a challenging field of study due in large part to both complicated nomenclature and lack of consensus on underlying pathogenesis. From a clinical perspective, multifocality is an issue of topography and largely divided into two groups: multifocal breast cancers (MFBC) defined as multiple foci of breast cancer in the same quadrant or within 3 to 5 cm of each other and multicentric breast cancers (MCBC) defined as multiple foci of breast cancer involving different quadrants of the breast or placed greater than 3 to 5 cm from each other [1-4].

Although this definition does not consider tumor biology, it has served the breast surgeons to make appropriate decisions on the extent of surgery vis-a-vis cosmesis, i.e. breast conserving therapy versus mastectomy. In addition, several studies have shown that MFBC often share histologic and immunohistochemical features that reflect relatedness [5-7]. This relationship has been further elucidated in several elegant studies using X-

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chromosome inactivation, cytogenetic studies using loss of heterozygosity and comparative genomic hybridization (CGH) to propose a clonal origin for these cancers [8-12]. Thus, it can be hypothesized that multifocality in invasive ductal carcinomas comes about by one of two mechanisms: through intramammary spread from a single index tumor or as a result of multiple independent primary foci, alternatively defining MFBC and MCBC, respectively.

We previously described a subset of multiple ipsilateral invasive ductal carcinomas that were associated with numerous satellite foci, higher lymphovascular invasion and nodal metastasis [13]. We hypothesized that these tumors spread throughout the breast via lymphatic channels akin to satellitosis/in-transit metastasis seen in melanoma *(hence satellitosis-phenotype)*.

There is evidence that at least a subgroup of multifocal breast cancers clinically behave more aggressively than unifocal breast cancers [14-20]. Therefore, the recognition of the aggressive subtypes of multifocal breast cancer may depend on the precise mechanism of multifocality. Herein, we sought to investigate the clonal relationship across separate foci of multifocal invasive ductal carcinoma using whole-exome sequencing (WES) in order to elucidate commonalities or lack thereof between both MFBC and MCBC.

2. Materials and methods

2.1. Case selection and immunohistochemistry

Six cases of multifocal invasive ductal carcinoma were selected from the de-identified previous study material [13]. These six cases include three cases of MBC-1 (satellitosis phenotype, Case 1-3) and three cases of typical MBC-2 (Case 4-6). Eighteen

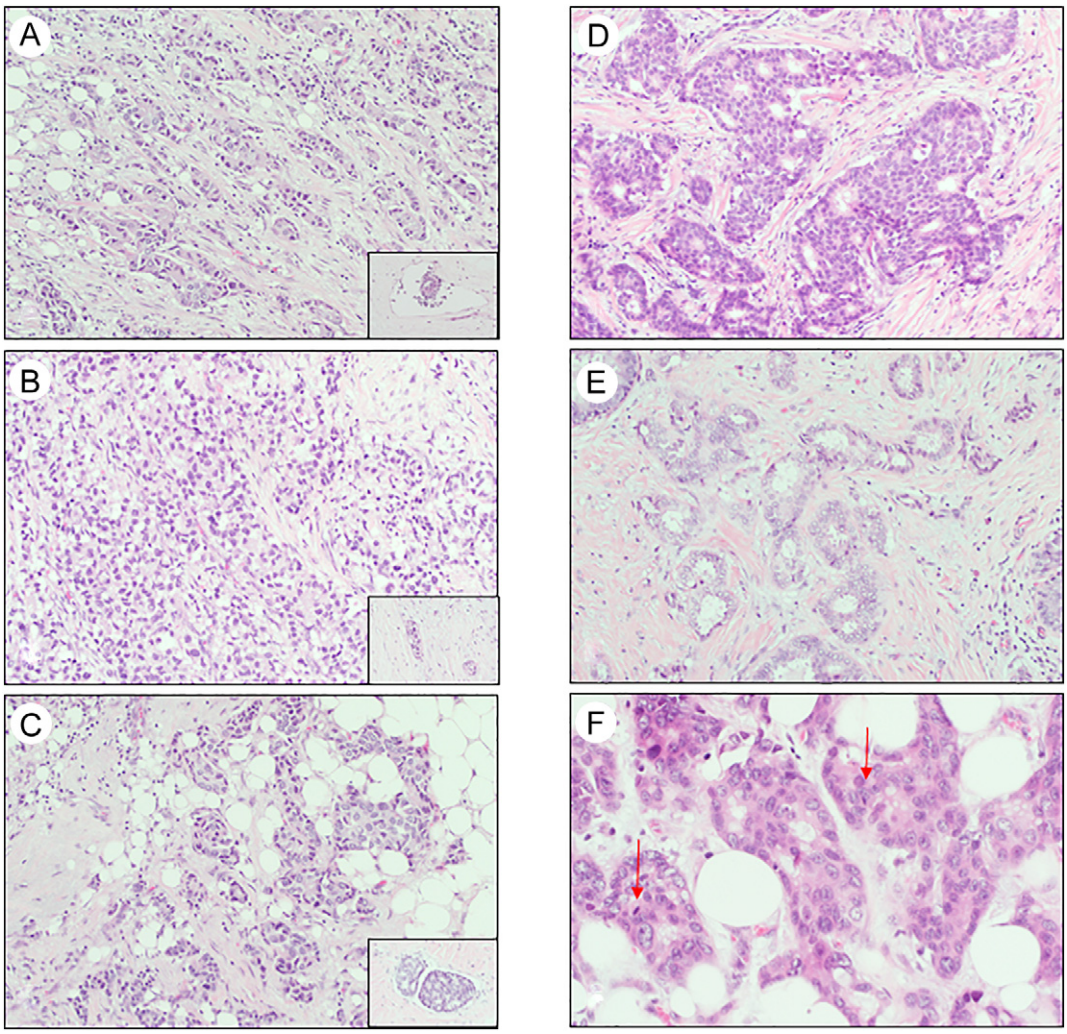

Fig. 1 *Case 1* (A-C): Invasive ductal carcinoma showing a solid architecture and grade 2 nuclei across all three foci (20× H&E). Ten separate foci of morphologically similar tumors were identified in this case (lymphovascular invasion was identified in each focus, inset; 20× H&E). *Case 4* (D-F): Invasive ductal carcinoma with cribriform architecture and grade 1 nuclei (T1, D, 20× H&E), glandular architecture and grade 2 nuclei (T2, E, 20× H&E) and mostly solid architecture with grade 3 nuclei and readily identifiable mitotic figures denoted by arrows (T3, F, 40× H&E). There were a total of four foci of morphologically distinct tumors in this case.

Table 1 Clinicopathological characteristics of MBC-1 and MBC-2 cases

Case	Age	Tumor foci	Histologic grade	ER/PR	*HER2*	Pathologic stage
1	65	10	2	+	-	pT1c,N2a
2	45	6	2	+	-	pT2,N1a
3	47	28	3	+	+	pT1c,N2a
4	45	4	2	+	-	pT1c,N0
5	70	4	2	+	-	pT1c,N0
6	59	3	3	+	-	pT2a,N2a

total tumor foci were analyzed across the 6 cases (3 foci per case). One benign lymph node was also microdissected per case as control. We defined MBC-1 as multiple foci of morphologically-similar invasive ductal carcinomas with only the index (largest) tumor containing ductal carcinoma in situ (DCIS). MBC-2 was defined as multiple morphologically similar or dissimilar foci with each focus harboring DCIS as previously described. We defined multifocality as at least 2 foci separated by normal breast tissue [13]. Immunohistochemistry (IHC) was performed on deparaffinized, rehydrated sections obtained from each representative focus from formalin-fixed, paraffin-embedded blocks using streptavidin-biotin-peroxidase epitope retrieval using Ventana automated system (Ventana, Tucson, AZ). Antibodies tested include the following: estrogen receptor (ER, clone SP1; Ventana, Tucson, AZ), progesterone receptor (PR, clone 1E2; Ventana, Tucson, AZ), human epidermal growth factor receptor 2 (Her2neu, clone 4B5; Ventana, Tucson, AZ). Our Institutional Review Board approved conducting this study.

2.2. Microdissection and DNA extraction

The tumor foci within each case were carefully outlined on the glass slides and separately microdissected along with a normal lymph node as control from six representative 10-μm-thick sections from formalin-fixed paraffin-embedded blocks. Individual tumor cellularity across foci was similar (mean cellularity 80%, Supplemental Material). Genomic DNA extraction was performed using QIAamp DNA FFPE Tissue Kit (QIAGEN).

2.3. Whole-exome sequencing and mutational analysis

Genomic DNA was subjected to whole-exome sequencing (xGen Exome Research Panel v1.0; Integrated DNA Technologies, Cat# 1056115) on Illumina HiSeq 4000 platform. The results were demultiplexed and converted to FASTQ format using Illumina bcl2fastq software. The reads were adapted and quality trimmed with Trimmomatic and then aligned to the human reference genome (build hg19/GRCh37) using the Burrows-Wheeler Aligner with the BWA-MEM algorithm according to methods previously described [21-23]. Low confidence mappings (mapping quality <10) and duplicate reads were removed using Sambamba software [24]. Further local indel realignment and base-quality score recalibration were performed using the Genome Analysis Toolkit. Single-nucleotide and small indel somatic variants were called with MuTect2. Detection of copy

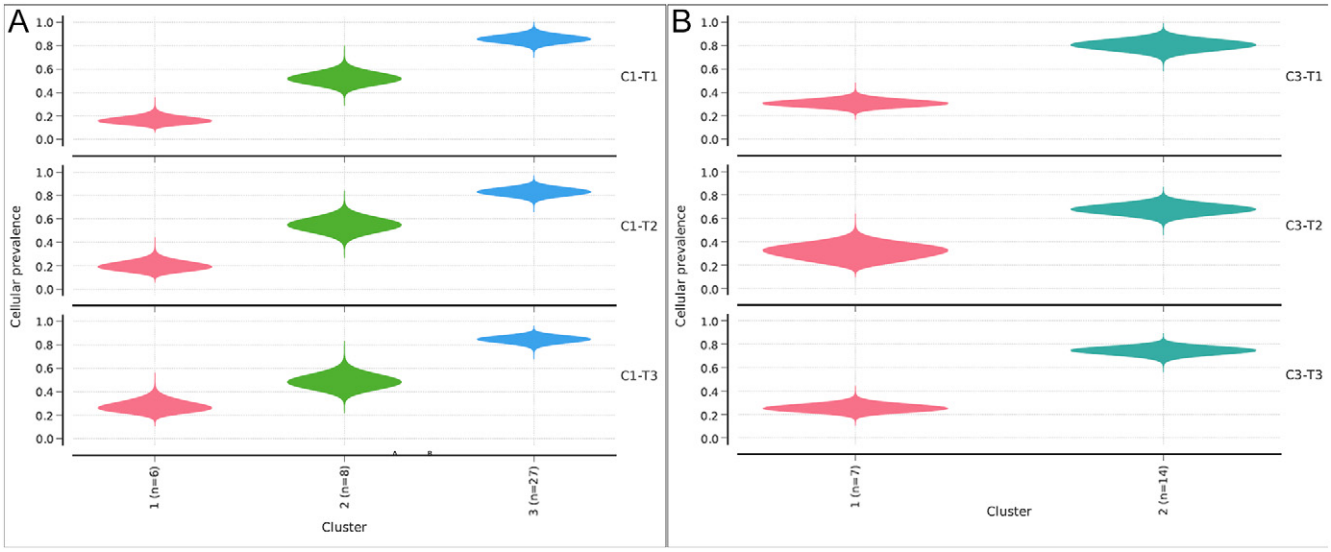

Fig. 2 Prevalence of clonal populations across foci using Pyclone. *Case 1* (A): Three distinct mutational clusters (1–3) are shared at similar variant allele frequencies across all foci containing 27 (blue), 8 (green) and 6 (red) events. The higher the score, the more prevalent is the cluster (set of genes/variants) within the tested tissue. *Case 3* (B): Two distinct mutational clusters are identified across all foci containing 14 (teal) and 7 (red) events.

number alterations and allelic imbalances were calculated using Control-FREEC according to methods previously described [25]. PyClone was used to convert the allelic frequency into a cellular prevalence score (CPS) to estimate the relative proportion of lesional cells harboring mutational clusters [26]. ANNOVAR was used to annotate variants with genomic context and functional consequence on genes as well as identify presence in public mutation and pathogenicity databases [27].

3. Results

3.1. Clinical features and histomorphology

All MBC-1 cases showed histologically similar foci with morphologic evidence of lymphovascular invasion (LVI) (Fig. 1A-C). In-situ carcinoma was present only in the largest focus of cancer (T1, not shown). In general, MBC-2 cases showed both histologically distinct and histologically similar foci, all with an associated in-situ carcinoma (Fig. 1D-F). Furthermore, MBC-2 cases lacked morphologic evidence of LVI except for Case 6. The clinicopathologic features are summarized in Table 1. MBC-1 cases on average engendered more cancerous foci (range, 6-28, average 14.6) when compared with MBC-2 (range, 3-4, mean 3.6). All cases were hormone receptor positive (ER+, PR+) and HER2 negative apart from Case 3 (*HER2* positive). In general, MBC-1 cases were associated with a higher nodal stage at presentation as well.

3.2. Whole exome sequencing and clonality analysis

The mean overall sequencing coverage for cases was 34×. Case 2 (MBC-1) was excluded from analysis due to low overall coverage. In order to investigate clonal evolution

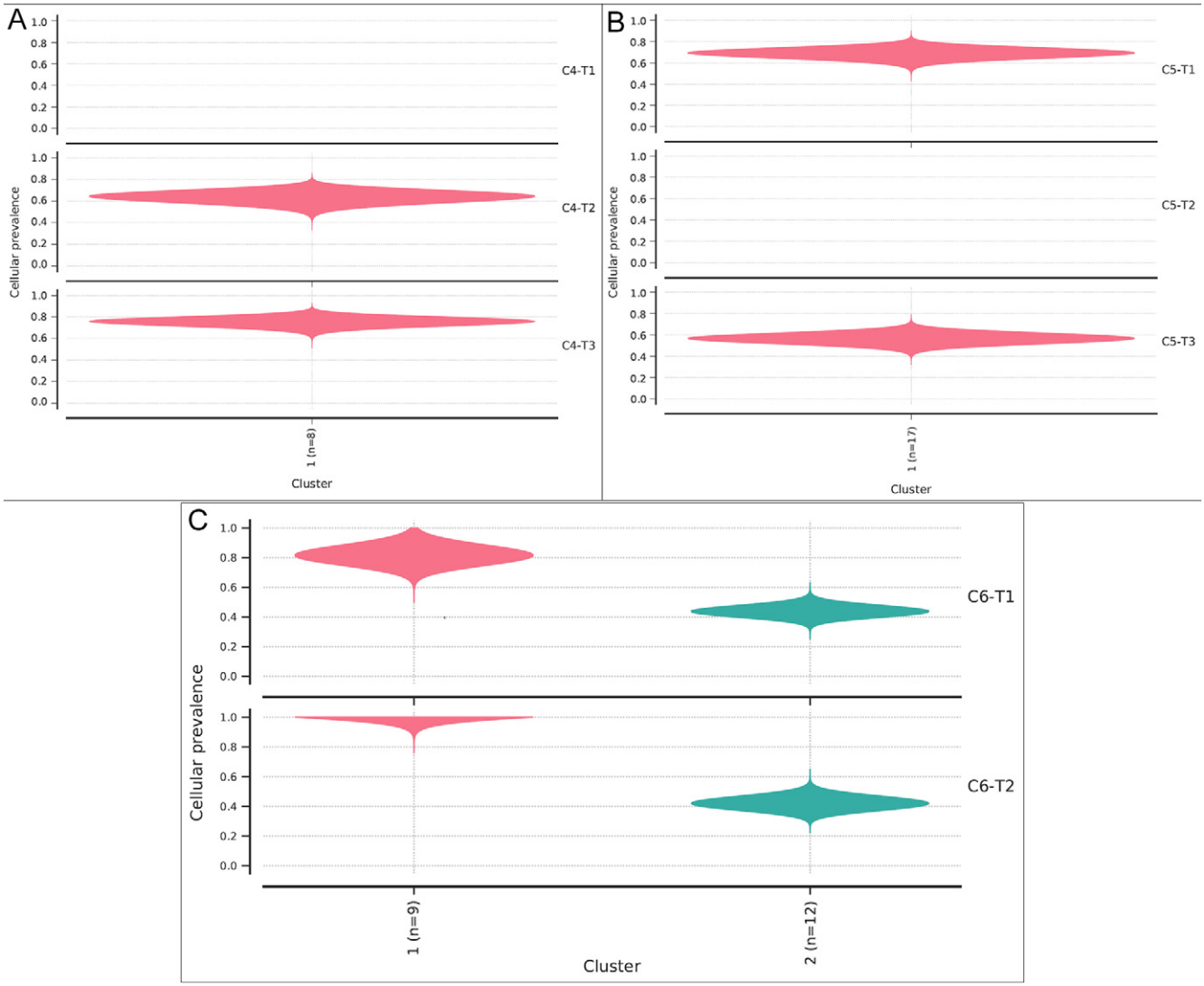

Fig. 3 *Case 4* (A): A single mutational cluster is shared amongst T2 and T3 foci, containing 8 (red) events. *Case 5* (B): A single mutational cluster is shared amongst T1 and T3 foci, containing 17 (red) events. *Case 6* (C): Two mutational clusters are shared amongst T1 and T2 foci, containing 12 (teal) and 9 (red) events, respectively.

between foci, a bioinformatics pipeline (Pyclone) was used to investigate clonality using genes with similar variant allele frequencies (VAF) to build distinct clusters. Briefly, clonality is derived from genes with similar VAF through a conversion of the VAF into a cellular prevalence (i.e. how frequently a particular variant occurs amongst a population of cells). Each cluster represents a single clone (26). All 5 cases showed between 1 to 3 genetic clusters, each harboring between 1 to 27 unique variants (Supplemental Material). Both MBC-1 cases showed gene clusters shared across all three tumor foci (T1-T3). Case 1 was defined by three clusters, with the largest cluster containing 27 distinct variants, defining a predominant clone with the highest cellular prevalence score (Fig. 2A). The additional clusters showed 8 and 6 distinct variants, respectively, shared across foci albeit at a lower cellular prevalence. Similarly, Case 3 showed two clusters shared by all three tumor foci; the predominant cluster contained 14 distinct variants with an additional cluster harboring 7 distinct variants (Fig. 2B). No variants were shared across the clusters within each respective case (data not shown).

Conversely, MBC-2 cases did not demonstrate a shared genetic cluster across all three foci. Case 4 shared a single cluster of 8 distinct variants, shared by two foci, T2 and T3 (Fig. 3A). A single cluster containing 17 distinct variants was shared by two foci, T1 and T3, in Case 5 (Fig. 3B). Case 6 contained two clusters containing 9 and 12 variants, shared by two foci, T1 and T2 (Fig. 3C). No variants were shared across the clusters within each respective case (data not shown).

Interestingly, in addition to shared genetic clusters, T1 and T2 foci in Case 6 also shared similar papillary-predominant histology, whereas the T3 focus exhibited a micropapillary morphology (Fig. 4A-C). Strikingly, the histology of lymph node metastasis was also papillary (Fig. 4D). These findings are suggestive of two distinct tumors with the papillary histology driving the clinical aggressiveness.

4. Discussion

The data herein represent the first study to our knowledge to examine clonality in MFBC using whole exome sequencing. The story and recognition of multifocal breast disease has now spanned centuries, with the first recognition by surgeons in the early 19th century followed by accurate histologic descriptions in the 1940s [5]. To date, careful molecular analysis has expanded our knowledge in understanding and discriminating MFBC as a single disease entity (clonal) or genetically different entities [1-11]. The biologic importance of differentiating two distinct disease processes has been an active matter of debate but evidence suggests MFBC is a more aggressive disease [13-19]. In our current study, we expand upon the biological behavior of a previously identified subgroup of invasive ductal carcinoma with high propensity for lymphovascular invasion, satellitosis and lymph node metastasis. This satellitosis phenotype tends to show strong clonal relationships, as evidenced by distinct

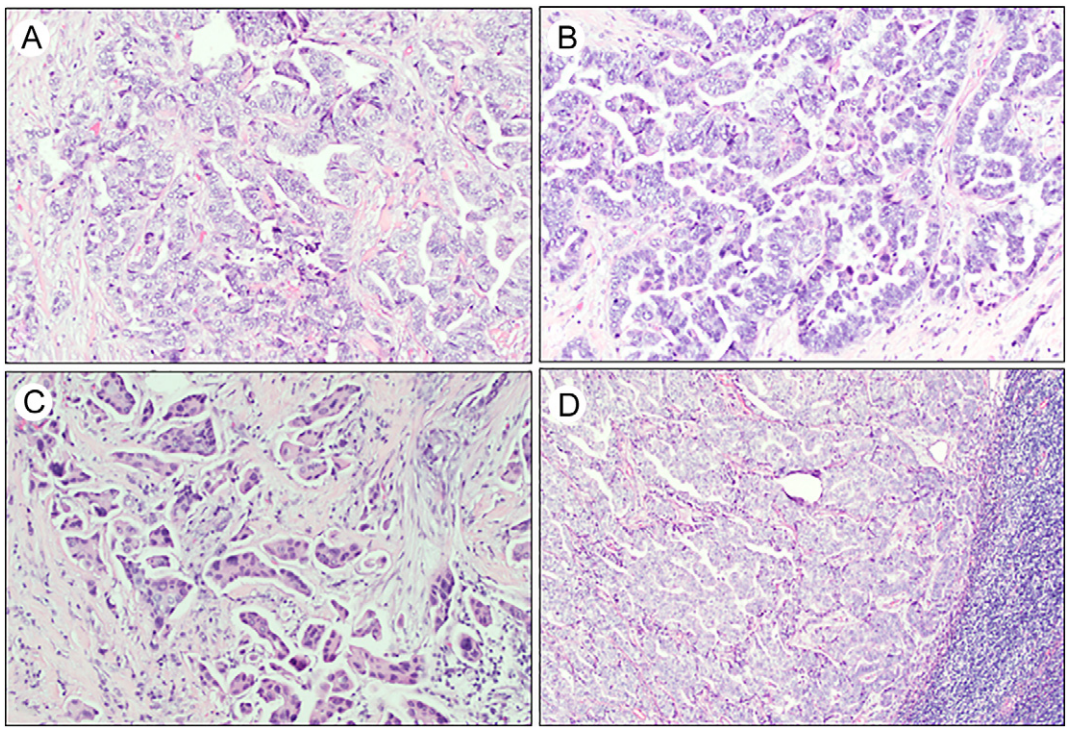

Fig. 4 *Case 6* (A-D): Invasive ductal carcinoma with T1 & T2 foci showing papillary morphology (A and B, respectively, 40× H&E). Micropapillary morphology is identified in the T3 focus (C, 40× H&E). Lymph node metastasis demonstrating papillary morphology (D, 20× H&E).

clusters of unique genetic variants that are shared across at least three foci. Conversely, our control cases of MFBC (MBC-2) seem to arise from multiple independent primaries and do not show strong evidence of clonality. The exception is case 6, which seems to be a hybrid case demonstrating two distinct tumor morphologies: papillary and micropapillary. The foci with papillary morphology showed strong clonal relationships (Fig. 3C). Surprisingly, the nodal metastasis showed papillary morphology similar to T1 and T2, despite the known propensity for lymph node spread in invasive micropapillary carcinoma (Fig. 4).

The current study adds to the growing armamentarium of techniques used to investigate the mechanism of MFBC, in that MFBC occurs through two distinct mechanisms (Table 2). Dawson and colleagues used immunohistochemistry and tumor histology to segregate intramammary spread of a single breast cancer from multiple synchronous tumors [7]. This was paralleled by cytogenetic studies using heterozygosity (LOH) on chromosome 16q and X-linked phosphoglycerokinase (PGK) gene inactivation to support clonal or divergent tumor origin [7,8]. Teixeira et al made seminal contributions to the MFBC literature by using CGH and subsequent unsupervised hierarchical clustering to delineate clonal relationships from multiple breast primaries in both ipsilateral and bilateral disease [9,10]. Most recently, human androgen receptor assay (HUMARA) was used to compare X-chromosome inactivation patterns in 71 cases of MFBC and found that most tumors (~90%) had the same X-chromosome inactivation pattern, suggestive of clonal origin [28]. Our findings corroborate this prevailing theory and further refine the definition of a subset of invasive ductal carcinomas with a predilection for clonal intramammary spread and lymph node metastasis. In the MBC-1 group, all tumor foci (T1-T3) showed similar cellular prevalence for the different clones/clusters identified in each of the studied tumors. These findings convey the similarity between different tumors and the fact that they represent clonally related tumor satellites.

Our study shows that whole-exome sequencing can be used as a tool to support a clonal origin in MFBC. However, it also brings to light several limitations. First, only two cases were assessed, as one case from our MBC-1 study group was excluded due to low overall sequencing coverage. This was likely due to poor sample condition owing to degradation of DNA from FFPE blocks. Another important caveat is the application of the Pyclone algorithm for clonal evolution in multifocal breast cancer. This application, while valid, might not be the most sensitive approach given that the timeframe for development of the foci is unknown. Indeed, the lack of an identifiable clone with similar variant allele frequency using Pyclone does not preclude the clonal nature of the tumor. Further studies using a larger number of cases with greater sequencing coverage would provide a more definitive and accurate assessment of clonality.

Insight into the pathogenesis of MBC-1 may be better suited using newer single cell RNA/DNA sequencing technologies, which could provide a more thorough understanding of cellular composition and relatedness amongst tumor foci. Further studies are currently underway in hopes of further unraveling the basis of this aggressive phenotype.

Table 2 Summary of studies examining multiple ipsilateral ductal carcinomas and techniques used (Adapted from Jain et al [1])

Year	Authors	Technique
1994	Dawson et al	Immunohistochemistry
1994	Noguchi et al	Loss of heterozygosity
1995	Tsuda and Hirohashi	X- linked phosphoglycerokinase (PGK) gene inactivation
1997	Teixeira et al	Comparative genomic hybridization
2004	Teixeira et al	Comparative genomic hybridization with hierarchical clustering analysis
2018	Kim et al	Humara X-chromosome inactivation
2019	Schwartz et al (current study)	Whole exome sequencing

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.08.021.

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