Original contribution

MAST2 and NOTCH1 translocations in breast carcinoma and associated pre-invasive lesions

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Summary There are several mutations and structural variations common to breast cancer. Many of these genomic changes are thought to represent driver mutations in oncogenesis. Less well understood is how and when these changes take place in breast cancer development. Previous studies have identified gene rearrangements in the microtubule-associated serine-threonine kinase (MAST) and NOTCH gene families in 5% to 7% of invasive breast cancers. Some of these translocations can be detected by fluorescence in situ hybridization (FISH) allowing for examination of the correlation between these genomic changes and concurrent morphologic changes in early breast neoplasia. NOTCH and MAST gene rearrangements were identified by FISH in a large series of breast cancer cases organized on tissue microarrays (TMA). When translocations were identified by TMA, we performed full cross-section FISH to evaluate concurrent pre-invasive lesions. FISH break-apart assays were designed for NOTCH1 and MAST2 gene rearrangements. Translocations were identified in 16 cases of invasive carcinoma; 10 with MAST2 translocations (2.0%) and 6 cases with NOTCH1 translocations (1.2%). Whole section FISH analysis of these cases demonstrated that the translocations are present in the majority of concurrent ductal carcinoma in situ (DCIS) (6/8). When DCIS wasn’t associated with an invasive component, it was never translocated (0/170, \( P = .0048 \)). We have confirmed the presence of MAST and NOTCH family gene rearrangements in invasive breast carcinoma, and show that FISH studies can effectively be used with TMAs to screen normal, pre-invasive, and coexisting invasive disease. Our findings suggest that these translocations occur during the transition to DCIS and/or invasive carcinoma.

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1. Introduction

Large genomic studies have identified numerous recurrent mutations and aneuploidies that can be found in the vast majority of breast cancers [1–3]. In contrast to invasive carcinomas, little is understood about the genomic changes associated with progression to breast cancer, from normal tissue to early neoplasias to carcinoma in situ to invasive carcinoma. However, it is likely that these...
genomic changes are important contributors to the process of carcinogenesis.

Prior research on pre-invasive breast neoplasia has largely focused on DNA copy number changes [4–6]; however, these studies did not have the current understanding of recurrent aneuploidies and did not focus on matched progression to invasive carcinoma. Very little work has been done on recurrent mutations in pre-invasive neoplasia. Some work has focused on general cancer hotspots, but other recurrent breast cancer–specific mutations have not been examined. With the exception of human epidermal growth factor receptor–2 (HER2) amplification, DNA copy number changes and the single nucleotide variations can be difficult to observe in situ. This is important as early breast neoplasia lesions are typically microscopic and can only be routinely observed in formalin-fixed paraffin-embedded material once a slide has been prepared [7].

A recent study found recurrent MAST (microtubule-associated serine-threonine kinase) and NOTCH family fusions in 5% to 7% of breast cancers [8]. Rearrangements were identified in the NOTCH1, NOTCH2, MAST1, and MAST2 genes, and these rearrangements were associated with increased oncogenic functional activity. Notch signaling is an evolutionarily conserved pathway that is essential for embryonic development, organogenesis, and tissue homeostasis. Aberrant Notch signaling is associated with several inherited developmental diseases and various types of cancer [6]. Clinical evidence supports a pro-oncogenic function for Notch signaling in several solid tumors, especially in breast cancer. Notch inhibitory agents, such as gamma-secretase inhibitors, are being investigated as candidate cancer therapeutic agents in a number of organ systems, and are becoming more and more relevant in the clinical setting [6,9,10].

Less is known about the MAST family of genes. They are thought to play a role in normal cell division, and alterations have reportedly produced a number of mitotic abnormalities, including spindle malformation, chromosome missegregation, centrosome amplification, and failure of cytokinesis [11]. This sort of chromosomal instability is common to carcinoma, and may help explain the role of MAST genes in carcinogenesis.

We sought to identify the previously described rearrangements in the MAST and NOTCH genes in a large number of breast cancer cases and to determine the extent, if any, to which concurrent pre-invasive lesions harbor these translocations. For this purpose we utilized fluorescence in situ hybridization (FISH), which allows for the identification of translocations with morphologic correlation. When rearrangements were identified in carcinoma, we went back to the primary tissue and analyzed whole section FISH preparations to evaluate for the absence or presence of involvement of earlier lesions such as ductal carcinoma in situ (DCIS), atypical ductal hyperplasia (ADH), flat epithelial atypia (FEA), columnar cell change (CCC), and finally normal breast epithelium where available.

2. Materials and methods

Tumor and normal samples were collected in compliance with the Health Insurance Portability and Accountability Act and approved by the Stanford University Medical Center Institutional Review Board.

2.1. Tissue microarrays

Interchromosomal translocations and large intrachromosomal translocations are easily identified by FISH in archival material. This approach allows for the correlation of morphologic features with these genomic changes. This enables the large scale screening of breast cancer tissue microarrays (TMAs) for these events and the examination of other types of breast neoplasia that are not easily obtained as fresh frozen material. We evaluated NOTCH1 and MAST2 gene rearrangements in 3 separate TMAs containing breast neoplasia. These included 283 cases of invasive carcinoma (TA-221), 285 cases of DCIS (TA-239), and 115 cases of invasive carcinoma (TA-241) that are from patients of the DCIS array. TA-239 contains 170 cases of pure DCIS without associated invasive disease, and 115 cases of DCIS that was associated with invasive disease, represented by TA-241. These cores represent material from 568 patients, with 20% of patients represented by two cores, and the remaining patients represented by a single core. The TMA was produced manually using the Manual Tissue Arrayer MTA-1 by Estigen Tissue Science (Tartu, Estonia). TMAs also included several tissue types for control purposes. The control tissues utilized included normal breast, placenta, appendix, adrenal, bladder, epididymis, liver, muscle, gallbladder, kidney, esophagus, lung, skin, seminal vesicle, pancreas, salivary gland, and prostate. The TMAs were divided into quadrants, with each quadrant having control tissues placed at the 4 corners, with additional controls placed diagonally through the center of each quadrants’ long axis. Using 2-color break-apart probes with FISH, we evaluated 1656 tissue cores (0.6 mm in diameter each).

2.2. Fluorescence in situ hybridization

Sections (4 μm thick) of the TMA slides were pretreated as described [12].

Locus-specific FISH analysis was performed by using the following bacterial artificial chromosomes (BACs) from the Human BAC Library RPCI-11 (BACPAC Resources Centre, Children’s Hospital Oakland Research Institute, Oakland, CA) and CTD (Caltech-D BAC library) clones from Invitrogen (Grand Island, NY) listed centromeric to telomeric: RP11-112P19, CTD-2310H10 (MAST2), and RP11-83N9, CTD-3213A21 (NOTCH1), and CTD-2182G14,RP11-713O7, RP11-1115, RP11-153P24 (MAST1), and RP11-671M21, CTD-2574B15, CTD-
2137J2, RP11-439A17, CTD-2071P15, CTD-3115K23, RP11-4N19 (NOTCH2). The successful probes, which were applied in pairs, are listed here centromeric to telomeric: RP11-112P19, CTD-2310H10 (MAST2), RP11-83N9, CTD-3213A21 (NOTCH1) (see Fig. 1). BACs were directly labeled with either Alexa fluor 647 DUTP (Invitrogen) or Cy3 dUTP (Amersham). The chromosomal locations of all BACs were validated by using normal metaphases. Probe labeling and FISH was performed by using Abbott Molecular reagents according to the manufacturer’s protocols. Slides were counterstained with DAPI for microscopy. For all slides, FISH signals and patterns were identified on an Ariol Imaging System (Leica Microsystems). Images were captured by using the Ariol software (Leica Microsystems) and were interpreted manually.

2.3. Scoring fluorescence in situ hybridization

When the majority (>50%) of a morphologically distinct cellular population expressed abnormal copy numbers of either probe, had loss of expression of one probe color, or had separation between probes more than two times that identified in control tissues, the FISH pattern was considered abnormal. These cases were further analyzed by whole section FISH.

2.4. Whole section fluorescence in situ hybridization

When TMA FISH results indicated an abnormality, original formalin-fixed paraffin-embedded (FFPE) blocks were obtained (where available), and FISH was performed on whole cross sections of the original material (n = 15). This tissue was hybridized, entirely imaged, and analyzed. Hematoxylin and eosin (H&E)–stained slides were also reviewed by two pathologists to identify the spectrum of lesions present on the specimen slides, and to correlate these areas on the imaged FISH specimen.

3. Results

Following on the earlier work of Robinson et al [8], we evaluated whether NOTCH1, NOTCH2, MAST1, and MAST2 gene rearrangements could be detected by FISH in archival material. The previously reported instances of translocations involving these genes included a mixture interchromosomal translocations, large intrachromosomal translocations, and small intrachromosomal translocations. The latter are difficult to detect by FISH break-apart assays as the separation between probes in a balanced translocation event (or inversion) is too small to be differentiated from the natural variation of probe separation in the untranslocated state. We successfully designed two-color break-apart probe sets (5′ and 3′ of the target gene) for the detection of NOTCH1 and MAST2 translocations. However, despite the use of multiple probe sets, we were unable to identify BAC probes that could detect translocations in either MAST1 or NOTCH2 (see Fig. 1).

We first aimed to confirm the presence of NOTCH and MAST gene rearrangements in breast carcinoma, both invasive and in situ, in 3 separate TMAs containing 568 patients with breast neoplasia represented by 1656 tissue
Fig. 2  Positive findings in invasive micropapillary carcinoma with H&E at 10× (A), 60× (B), and MAST2 FISH (C) with amplification of red signal. Concurrently identified intermediate grade DCIS with H&E at 10× (D), 60× (E), and MAST2 FISH (F) with amplification of red signal.

Fig. 3  Amplification of NOTCH1 identified in high-grade DCIS with uninvolved surrounding stroma. H&E stained tissue at 10× (A), and 60× (B) magnifications. High-power NOTCH1 FISH images without (C) and with labels (D). In panel D, DCIS is present to the right of the dashed red line, and uninvolved stromal cells are to the left of the line.
cores. We identified 1398 interpretable FISH hybridizations (representing 501 patients) for the MAST2 and NOTCH1 genes, an overall 88.6% success rate. Hybridizations were considered failed for a number of reasons including direct loss of tissue from array slides, failure of probe hybridization, and uninterpretable hybridizations, typically due to high background of the fluorescent signal. Images of these cores were then manually reviewed for translocations of the two-color break-apart probes. Positivity was defined as selective loss of either probe, or a balanced translocation within a definable cell population, as demonstrated by fixed separation between probes.

We identified 16 unique cases for whole section analysis (overall 3.2% of patients; see Table 1). Of these 16 cases, tissue blocks were available for 11. When these cases were evaluated by whole section FISH, all were again found to be positive in the invasive component (overall 2.2% of patients). All of these cases were invasive ductal carcinoma (IDC), with 1 infiltrating micropapillary type, and 10 having no special type. All cases were estrogen receptor (ER) positive, all but 1 case were progesterone receptor (PR) positive, and all were reported as HER2 negative (see Table 2).

We then evaluated the tissue surrounding the translocation-positive carcinomas for the presence of the same translocation in pre-invasive lesions. This was done by reviewing whole section FISH for the selected translocation-positive cases. H&E stained slides that contained representative areas of carcinoma on the TMA core were reviewed for each case. We identified pre-invasive lesions on these slides and performed whole section FISH to assess for the presence of the identified translocations (See Fig. 2). Consecutive H&E-stained slides were also used as guides when interpreting morphology on the FISH imaged slides. For the identification of pre-invasive lesions, we used well-established diagnostic criteria[13]. Carcinoma in situ was identified in 8 of the 11 cases. Of these 8 cases, translocations were identified in 6 (4 of 5 for MAST2, 2 of 3 for NOTCH1, see Fig. 3 and Table 2). When DCIS was involved, it was always involved in cases that also had invasive disease (the 6 positive cases were a subset of 115 samples with concurrent invasive disease; no positive cases were identified in the 170 cases of pure DCIS without associated invasion, p = 0.0048). Even earlier lesions (ADH, FEA, CCC) were identified in 8 of the 11 cases. One of these

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**Fig. 4**  H&E-stained invasive carcinoma (A), with matching NOTCH1 FISH study showing selective amplification of the red probe (B). Columnar cell change stained with H&E (C), showing heterogeneity with FISH showing a balanced translocation in some cells (indicated by two signals widely separated, yellow arrow), with other cells showing normal signal pattern (red arrow) (D).
earlier lesions showed FISH heterogeneity with evidence of a subpopulation harboring a balanced translocation (CCC with the NOTCH1 gene, see Fig. 4).

4. Discussion

Recent sequencing studies have shown that breast cancer–specific aneuploidies and single nucleotide variations in breast cancer are common and vary in incidence among breast cancer subtypes [1–3, 14]. A few aneuploidies are present in a large subset of breast cancers, such as gain of chromosome 1q, MYC amplification, and 16q deletion. Recurrent single nucleotide variations have frequencies that range from 80% involvement of TP53 mutations in basal-like, 40% involvement of PIK3CA in luminal, and 10% involvement of GATA3 overall [3].

Much less is known about genomic changes in pre-invasive breast neoplasia. Prior studies have found aneuploidies to be present in a spectrum of lesions ranging from ADH to DCIS [4, 7, 14–16]. Our recent study of whole-genome sequencing of matched proliferative early neoplasia to DCIS to IDC has found that aneuploidies can be among the earliest recurrent events in breast cancer development [11]. Another of our recent studies on PIK3CA shows that single nucleotide variations in this gene can be found in proliferative early neoplasia (like CCC and ADH) and carcinoma in situ [17]. The MAST and NOTCH gene families exhibit a much lower incidence of translocations in breast carcinoma, and to our knowledge haven’t been studied in pre-invasive lesions to date. Using FISH, translocation can be easily detected in sections of archival material creating the opportunity to assess for the presence of these events in small pre-invasive early breast neoplasias.

Table 1  Summary of results from initial survey of TMAs and secondary survey of whole section FISH studies

<table>
<thead>
<tr>
<th>Initial survey of TMAs</th>
<th>Secondary morphological survey of full-section FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surrounding tissue in available blocks</td>
</tr>
<tr>
<td>Total patients</td>
<td>565</td>
</tr>
<tr>
<td>Patients with successful hybridization</td>
<td>501</td>
</tr>
<tr>
<td>MAST2 hybridized</td>
<td>440</td>
</tr>
<tr>
<td>Positive cases</td>
<td>10</td>
</tr>
<tr>
<td>Blocks available</td>
<td>6</td>
</tr>
<tr>
<td>NOTCH1 hybridized</td>
<td>476</td>
</tr>
<tr>
<td>Positive cases</td>
<td>6</td>
</tr>
<tr>
<td>Blocks available</td>
<td>5</td>
</tr>
<tr>
<td>Overall FISH-positive cases</td>
<td>16 (3.2%)</td>
</tr>
</tbody>
</table>

Table 2  Histologic, hormonal, and HER2 characteristics of cases with translocations identified in invasive carcinoma (n = 11). Also listed is the status of in situ and earlier lesions for these cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Gene translocated</th>
<th>Lesion Type</th>
<th>Grade</th>
<th>Hormone status</th>
<th>HER2 status</th>
<th>In situ Types</th>
<th>Translocation status</th>
<th>Earlier lesions Types</th>
<th>Translocation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MAST2</td>
<td>IDC</td>
<td>2</td>
<td>ER/PR+</td>
<td>1+ IHC</td>
<td>IG DCIS</td>
<td>Translocated</td>
<td>ADH, CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>MAST2</td>
<td>Micropapillary IDC</td>
<td>2</td>
<td>ER+</td>
<td>2+ IHC, Neg IHC</td>
<td>IG DCIS</td>
<td>Translocated</td>
<td>FEA</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>MAST2</td>
<td>IDC</td>
<td>1</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>IG DCIS</td>
<td>Normal</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>MAST2</td>
<td>IDC</td>
<td>3</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>None</td>
<td>NA</td>
<td>ADH, CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>MAST2</td>
<td>IDC</td>
<td>2</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>LG DCIS</td>
<td>Translocated</td>
<td>CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>MAST2</td>
<td>IDC</td>
<td>3</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>IG DCIS</td>
<td>Translocated</td>
<td>CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>NOTCH1</td>
<td>IDC</td>
<td>1</td>
<td>ER/PR+</td>
<td>2+ IHC, Neg IHC</td>
<td>None</td>
<td>Translocated</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>NOTCH1</td>
<td>IDC</td>
<td>1</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>None</td>
<td>NA</td>
<td>CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>NOTCH1</td>
<td>IDC</td>
<td>2</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>HG DCIS</td>
<td>Normal</td>
<td>CCC</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>NOTCH1</td>
<td>IDC</td>
<td>1</td>
<td>ER/PR+</td>
<td>Neg IHC</td>
<td>None</td>
<td>NA</td>
<td>CCC</td>
<td>Translocated</td>
</tr>
<tr>
<td>11</td>
<td>NOTCH1</td>
<td>IDC</td>
<td>2</td>
<td>ER/PR+</td>
<td>0+ IHC</td>
<td>IG DCIS</td>
<td>Translocated</td>
<td>None</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: ER, estrogen receptor; HG, high grade; IG, intermediate grade; IHC, immunohistochemistry; LG, low grade; NA, not applicable; Neg, negative; ADH, atypical ductal hyperplasia; CCC, columnar cell change; FEA, flat epithelial atypia; IDC, invasive ductal carcinoma; FISH, fluorescence in situ hybridization.
We evaluated MAST and NOTCH gene family translocations in a large number of breast carcinomas with the use of TMAs. Using a FISH break apart approach against the target genes, we were able to screen a large number of cases, select out for potential positives, and perform whole section FISH analysis to examine a variety of pre-invasive lesions including carcinoma in situ, CCC, FEA, and ADH. As with any study of this type, tissue heterogeneity and appropriate sampling is an issue. It is possible our screening method missed certain cases with heterogeneous gene involvement, a factor that warrants further study. However, our full cross-section analysis finds that the translocation is present in the vast majority of carcinoma cells, if not all of them, making this issue less likely to have affected our results.

When all cases are combined, out of the 501 patients with cores that successfully hybridized, we found 11 cases with either MAST2 or NOTCH1 translocations (2.2% overall) in invasive carcinoma. Six of 8 cases tested had an identifiable translocation concurrent in adjacent carcinoma in situ, suggesting involvement is common at the DCIS stage. One case of columnar cell change also demonstrated cells with translocation (1 of 8 cases with columnar cell change), though this finding was heterogeneous within the lesion and was an extremely rare occurrence given that CCC occurred multiple times throughout the slide in several cases. No case had abnormal FISH results in both the MAST and NOTCH genes. No abnormalities were present in surrounding normal breast terminal duct lobular units, with all having greater than 100 normal luminal cells.

Identification of pre-invasive genetic abnormalities is important not only for prognostic purposes, but to further the understanding of the neoplastic process. The HER2 gene, associated with significant prognostic implications in invasive carcinoma, has been shown to be involved in the progression of early lesions in a subset of breast neoplasia. In one study, half of the cases of ADH were amplified (7 of 13 cases), with more positive cases identified in higher-grade lesions (21:22 cases of DCIS) [18]. With the majority of in situ carcinoma already being HER2 amplified, the authors concluded HER2 is mainly involved in the initiation of oncogenesis, with unknown influence on tumor progression. Further supporting this theory, HER2 has been shown to promote the dissemination of incompletely transformed malignant cells, being part of the metastatic process 5 years before a clinically apparent carcinoma is identifiable [5]. Though the MAST and NOTCH genes have not yet been shown to be associated with such phenomena, it is curious that we have identified their change in such early lesions as well. Even more interesting, every time we identified a translocation in DCIS, it was associated with neighboring invasive disease which also exhibited translocations in every case. No cases of DCIS without associated invasive disease (n = 170) showed any abnormality (P = 0.0048). This suggests the MAST and NOTCH genes may play a similar role in the carcinogenic process to other well described genes that are often found altered in pre-invasive disease.

This would include genes such as HER2, retinoblastoma (RB), Hypoxia inducible factor-1α (HIF-1α), MYC, COX2 [5,19–21].

We have shown MAST2 and NOTCH1 gene translocations can be identified in breast carcinoma, and when using FISH as the primary detection method, we find these at a lower frequency than previously reported. Detecting these translocations is highly dependent upon the detection technique utilized, and the difference observed in our study highlights this. The original paper utilized paired-end transcriptome sequencing [8], and we undoubtedly sacrificed a degree of sensitivity to pursue analysis of earlier lesions within our samples. Little is known about the demographic distribution of these translocations as well, and it is possible that our patient population exhibits a different frequency of involvement when compared to the previously mentioned paper, which utilized a combination of local and international samples, as well as commercially available cell lines. This later theory is supported by the lesion specifics identified in our two papers. All of our positive cases were estrogen receptor positive, at odds with the original paper where ER positivity was absent in their NOTCH1 translocations (0/6), and present in half of the MAST2 translocations (1/2). We cannot explain this variance, but perhaps it emphasizes the difference between the samples studied. If you ignore the cell lines from the original study and only focus on tumor samples, all of their MAST2 translocations were ER positive (1/1), concordant with our tumor samples.

With the use of FISH on TMAs containing formalin-fixed, paraffin-embedded tissue, translocations can be identified in earlier lesions than invasive carcinoma, certainly including in situ carcinoma, and even potentially in pre-neoplastic states. The development of early neoplasia is an increasingly popular focus in oncogenetic research, and this study serves as a proof of principle regarding the use of FISH to evaluate pre-invasive tissues in malignant samples. In the future, studies like this that are specifically designed to evaluate pre-invasive lesions may provide evidence to suggest drug targets that are present at early stages of tumor development. This could enable therapeutic interventions designed to target not only the carcinoma, but the reservoir of related cells from which the carcinomas derive [14].

References


