A Fluorescence In Situ Hybridization Study of ETV6-NTRK3 Fusion Gene in Secretory Breast Carcinoma

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The translocation t(12;15)(p13;q25), in which the ETV6 gene from chromosome 12 is rearranged with the NTRK3 gene from chromosome 15, has recently been identified in secretory breast carcinoma (SBC). This fusion gene was initially described in congenital fibrosarcoma and congenital mesoblastic nephroma. The biological consequence of this translocation is the expression of a chimeric protein tyrosine kinase with potent transforming activity. To assess the frequency of t(12;15)(p13;q25) in breast cancer, we developed complementary probe sets (fusion and split-apart probes) for the detection of this translocation by fluorescence in situ hybridization (FISH) in paraffin-embedded, formalin-fixed tissue sections. We tested four histologically confirmed cases of SBC for the presence of the ETV6-NTRK3 gene fusion and then applied the FISH assay to tissue microarrays (TMAs) in order to screen 481 cases of formalin-fixed, paraffin-embedded invasive breast carcinomas of various histologic subtypes. Three of the four cases of SBC revealed fusion signals. Of the 481 cases in the TMAs, 202 gave signals of sufficient quality for screening by FISH, and only one case showed fusion signals in most or all of the tumor cells. On review of the histology of this case, SBC was confirmed. On the other hand, none of the fusion-negative breast cancers revealed SBC histology. In all cases, the results from the fusion and split-apart FISH assays for the ETV6-NTRK3 fusion genes were concordant. Our data suggest that the ETV6-NTRK3 fusion gene is a specific genetic alteration in SBC.

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FISH assay for the detection of ETV6-\(NTRK3\) fusion gene have a locally aggressive behavior, are usually of low histologic grade, and rarely metastasize, consistent with the clinical features of SBC (Knezevich et al., 1998a). Fluorescence in situ hybridization (FISH) was attempted in 13 cases of SBC, 12 of which had tested positive for the \(ETV6-\)NTRK3 fusion transcript by RT-PCR. All nine cases that gave interpretable FISH results were positive for the gene fusion according to FISH analysis; the other four cases, including the RT-PCR-negative case, were uninterpretable (Tognon et al., 2002).

One aim in this study was to develop a robust FISH assay for the detection of \(ETV6-\)NTRK3 gene fusion in formalin-fixed archival tissue samples and to determine the sensitivity and specificity of this new assay by analysis of a large series of archival infiltrative breast cancers and four known cases of SBC. The use of tissue microarrays (TMAs) is a useful technique for high-throughput analysis of large numbers of cancer samples on a single slide by either immunohistochemistry or FISH (Kallioniemi et al., 2001). In our present study, we used TMAs for FISH screening of t(12;15) in breast cancer.

Initially, 481 primary, female breast cancers (sequential archival cases from Vancouver General Hospital, British Columbia, Canada, from 1974 to 1995) were identified. For tissue array construction, representative areas of invasive carcinoma were selected and marked on a hematoxylin and eosin (H and E)–stained slide and its corresponding tissue block. The TMAs were assembled by use of a tissue-arraying instrument (Beecher Instruments, Silver Spring, MD), as described previously (Makretsov et al., 2003). Two 0.6-mm-diameter tissue cores were taken from each case. Three composite high-density tissue microarray blocks were constructed, and serial 6-\(\mu\)m sections were cut with a Leica microtome and transferred to adhesive-coated slides. One section from each tissue array block was stained with H and E. The presence of tumor cells, stromal elements, and inflammatory infiltrates was assessed on these sections prior to interpretation of the FISH assay in order to reduce misinterpretation of hybridization signals.

Four cases of formalin-fixed, paraffin-embedded SBC referred for pathology review by the breast pathologist of the British Columbia Cancer Agency were also subjected to FISH analysis. Clinical follow-up data were obtained from the central cancer registry of the BC Cancer Agency.

In a previous study, dual-color split-apart probes for \(ETV6\) (exons 1 and 8) and differentially labeled fusion probes for a 12p13 breakpoint-spanning YAC \((ETV6)\) and a 15q25 breakpoint-spanning YAC \(NTRK3\) were used in FISH analysis of t(12;15)(26). In the current study, three dual-color FISH assays—a t(12;15) translocation fusion probe assay, a chromosome 12 \((ETV6)\) split-apart probe assay, and a chromosome 15 \((NTRK3)\) split-apart probe assay—were used for detecting the t(12;15) translocation in tumor samples (Fig. 1). All BAC clones used in this study were obtained from the BACPAC Resources Centre at the Children’s Hospital Oakland Research Institute. All probes were labeled by nick translation with use of the manufacturer’s recommended protocol (Invitrogen Canada Inc., Burlington, Ontario, Canada). BAC clones RP11-434C1 and RP11-407p10 (probe A), telomeric to \(ETV6\) on 12p, were labeled with spectrum orange, and clones RP11-525I3 and RP11-267J23 (probe B), centromeric to \(ETV6\), were labeled with spectrum green. On chromosome 15, BAC clones RP11-247E14 and RP11-893E1 (probe D), telomeric to \(NTRK3\) on 15q, were labeled with spectrum orange, and clones RP11-114I9 and RP11-730G13 (probe C), centromeric to \(NTRK3\), were labeled with spectrum green. The three probe sets (encoded AB, CD, and AC, Fig. 1A–C) were initially applied to 6-\(\mu\)m formalin-fixed paraffin tissue sections from a previously published \(ETV6-NTRK\) fusion-positive case (Tognon et al., 2002) and produced the expected fusion or break-apart signals in >80% of cells. Furthermore, fusion or break-apart signals were observed in <5% of normal cells from peripheral-blood interphase preparations and in <10% of normal breast epithelia and in stromal and inflammatory cells within the tissue cores in the TMAs. Initial FISH analysis with a fusion probe set (A:C) were scored negative if less than 10% of cancer cells had clear dual-color positive fusion signals and positive if >50% of the cancer cells had clear fusion signals. Equivocal cases, those with between 10% and 50% fusion signal–positive cancer cells, would be screened again with one of the two break-apart FISH assays (A:B or C:D). Those would be considered positive if >30% of the cancer cells had clearly identifiable single-color signals.

originally cloned in pediatric mesenchymal cancers (congenital fibrosarcoma and cellular mesoblastic nephroma) (Knezevich et al., 1998a, 1998b). This gene fusion is the result of a reciprocal translocation, t(12;15)(p13;q25). It encodes a chimeric tyrosine kinase with potent transforming activity in fibroblasts. All pediatric tumors bearing the \(ETV6-\)NTRK3 fusion gene have a locally aggressive behavior, are usually of low histologic grade, and rarely metastasize, consistent with the clinical features of SBC (Knezevich et al., 1998a). Fluorescence in situ hybridization (FISH) was attempted in 13 cases of SBC, 12 of which had tested positive for the \(ETV6-\)NTRK3 fusion transcript by RT-PCR. All nine cases that gave interpretable FISH results were positive for the gene fusion according to FISH analysis; the other four cases, including the RT-PCR-negative case, were uninterpretable (Tognon et al., 2002).

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Six-micrometer tissue and TMA sections were baked overnight at 65°C and then subjected to FISH with a modified protocol (Vysis, Downers Grove, IL) (Hughes-Davies et al., 2003). Slides were kept at 4°C until scored. FISH signals were analyzed with a Zeiss Axioplan epifluorescence microscope equipped with a COHU-CCD camera. Images were captured with Metasystems ISIS software (MetaSystems Group, Inc., Belmont, MA) with seven focal planes stacked for the analysis of each FISH image.

All cases included in the tissue array were analyzed by FISH with the fusion probe set without prior knowledge of the histologic subtype of the tumor. Satisfactory hybridization signals and sufficient tissue (at least 40 tumor cell nuclei per tissue core) were present in 202 of the 481 cases included in tissue arrays. The remaining cases were excluded for the following reasons: core loss during sectioning or FISH analysis, presence of less than 40 clearly identifiable tumor cells in the cores from any case, and failed hybridization. Merged dual-color FISH signals, indicating the presence of ETV6-NTRK3 gene fusion, were detected in one case (Fig. 2, 1A–C). The other 201 cases showed normal signals or less than two fusion signals per field ($\times$1,000) and were considered to be fusion-negative.

Histologic typing of whole H and E sections was performed without prior knowledge of FISH data. Only one of the 202 breast cancers with FISH data was classified as SBC, which was the ETV6-NTRK3 fusion-positive case detected by FISH (Table 1, case 1). The other 201 cases were classified as follows: IDC, NOS (180 cases), infiltrative lobular carcinoma (14 cases), mucinous carcinoma (4 cases), signet ring carcinoma (1 case), papillary carcinoma (1 case), and medullary carcinoma (1 case). None of the ETV6-NTRK3 FISH-negative cases showed classical SBC histology.

To confirm our initial findings, we retested whole sections from the case identified as fusion-positive on the TMA and four additional archival cases of SBC with the three complementary probe

Figure 1. Schematic representation of the FISH assay designs. (A) Normal chromosome 12. (B) Normal chromosome 15. (C) der(15) t(12;15) Chromosome. Probes A (RP11-434C1 and RP11-407p10), B (RP11-525I3 and RP11-267J23), C (RP11-114f9 and RP11-730G13), and D (RP11-247E14 and RP11-893E1) are BACs flanking the involved ETV6 (A+B) and NTRK3 (C+D) genes. Dotted lines represent breakpoints on chromosomes 12 and 15. (D) Interpretation of FISH using split-apart (A+B) and (C+D) and fused (A+C) probe sets. In the absence of t(12;15), all cells show both differentially labeled parts of the NTRK3 gene (C+D). In the presence of the translocation, cells show disrupted NTRK3 gene (C+D). In the absence of t(12;15), cells have two copies of ETV6 (green) and NTRK3 (red) (A+C). In the presence of t(12;15), cells show fused ETV6 and NTRK3 genes (merged green and red signals) as well as one normal copy of both genes (A+C).
sets (A:C, A:B, and C:D). Three of the five cases of SBC showed the presence of ETV6-NTRK3 fusion signals and both ETV6 and NTRK3 break-apart signals in the great majority of tumor cells; one case showed the presence of fusion and break-apart signals in >40% of tumor cells; and one case was negative (<10% of cells with fusion or break-apart signals). FISH data interpretation is represented graphically in Figure 1D. H and E and corresponding FISH images of cases 1–5 are presented in Figure 2. Clinicopathologic data on the five cases of SBC included in this study (one case identified in TMA and four additional cases) are shown in Table 1. Upon review of the H and E sections, all four cases exhibited classical SBC histology: tumors were chiefly comprised of a ductal proliferation containing intra- and extracellular accumulation of eosinophilic material. The epithelial cells were cuboidal, with round, uniform nuclei, and, with the exception of case 4, which was FISH-negative, had a low mitotic rate. Some cases exhibited extensive dense fibrosis of the tumor stroma in the central

**TABLE 1. Clinicopathologic Data on Five Cases of Secretory Breast Carcinoma**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age</th>
<th>Nottingham tumor grade</th>
<th>TNM stage</th>
<th>Follow-up, status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>30</td>
<td>1</td>
<td>T1N0M0</td>
<td>15 years, alive, no evidence of disease</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>19</td>
<td>1</td>
<td>T1N0M0</td>
<td>1 year, alive, no evidence of disease</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>72</td>
<td>1</td>
<td>T1N0M0</td>
<td>4.75 years, died of trauma</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>52</td>
<td>3</td>
<td>T1N1M0</td>
<td>8 months, alive, no evidence of disease</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>58</td>
<td>2</td>
<td>T1N0M0</td>
<td>7 years, died of colon cancer</td>
</tr>
</tbody>
</table>
part of the tumor nodule. There was no evidence of vascular invasion in any case.

SBC is a rare subtype of ductal breast cancer with a recurrent translocation t(12;15), resulting in the expression of the ETV6-NTRK3 chimeric oncoprotein (Knezevich et al., 1998a,b; Tognon et al., 2002). Consistent results of serial FISH experiments with either split-apart or fusion probe sets showed disruption of the ETV6 and NTRK3 genes and the presence of the ETV6-NTRK3 fusion gene in four of five SBC cases in our series. This is in agreement with the previous study of Tognon et al. (2002). Our screening TMA FISH results showed that the ETV6-NTRK3 translocation is restricted to the secretory breast carcinoma histotype and is not seen in other subtypes of breast cancer, including some subtypes that share similar histologic patterns with SBC, confirming the rarity of this event and the high specificity of the assays. Two subtypes of breast cancer that have some overlap of histologic features with SBC, that is, signet ring carcinoma (one case in our series) and mucinous carcinoma (five cases), were fusion-negative.

We failed to detect the ETV6-NTRK3 fusion gene in one case of SBC, which, in contrast to the other cases in our series, had areas of higher histologic grade and lymph node metastases. Although this case may have had a cryptic t(12;15), it is unlikely that all three probe sets used would have been negative. Another explanation is that it was a pathologic phenocopy, or it is possible that the fusion was present in this cancer at earlier stages of tumor development but was lost during progression. RT-PCR failed in this case, probably because of the poor quality of the mRNA in the tissue block.

The rarity of SBC prevents investigators from doing direct cell culture and karyotyping in most cases; therefore, the FISH assay discussed above could serve as a useful ancillary diagnostic test for detection of this translocation in archival material. Although RT-PCR is highly specific for the detection of fusion transcripts, because of the poor quality of mRNA extracted from formalin-fixed, paraffin-embedded material, this method often requires multiple attempts or fails completely and thus can be more “tissue-consuming” than FISH. This is of critical importance in studying rare lesions when there is a limited amount of tissue available. The greater stability of tumor DNA compared to mRNA and the smaller tissue requirements may make FISH superior to RT-PCR for detection of translocations in archival tissue samples.

Based on the results of this study, the FISH-based protocol we used would have detected all fusion-positive SBC cases, including the previously published positive control, with minimal retesting and no false positives.

Earlier studies and meta-analyses of case reports suggested that SBC is associated with a more favorable outcome than are other more common types of invasive breast cancer, although that might reflect the age-dependent behavior of SBC, with an indolent course at a younger age that turns into aggressive behavior in older patients (Tavassoli, 1992). Although our series included only five cases of SBC and had only limited follow-up data, none of the patients in this series has died of breast cancer. A large multicenter study is needed to clarify the prognostic significance of SBC. In such a study, the FISH assays for the ETV6-NTRK3 fusion gene could be used as an objective diagnostic criterion for SBC. At this time, there is no explanation as to why this translocation is associated with a unique histologic subtype of breast cancer.

It is possible that the ETV6-NTRK3 fusion represents but one of several translocations that are important in the pathogenesis of breast cancer. A chromosomal rearrangement in the breast cancer–derived cell line MCF7 fuses two novel genes (BCAS3 and BCAS4) (Barlund et al., 2002), and a recurrent translocation breakpoint in breast and pancreatic cancer cell lines targets the neuregulin/NGR1 gene (Adelaide et al., 2003). In poorly differentiated nasopharyngeal carcinoma, a recurrent translocation, t(15;19)(q13;p13.1), fuses the BDR4 and NUT genes (French et al., 2003), and in a mucoepidermoid carcinoma, a t(11;19)(q21;p13) creates a novel fusion product that disrupts a Notch signaling pathway (Tonon et al., 2003). These observations suggest that recurrent chromosomal rearrangements are more common in solid cancers than previously believed. The role of recurrent chromosomal rearrangements in breast cancer pathogenesis is a focus of further studies. We propose that the application of translocation-specific FISH assays to TMAs is a powerful tool for the determination of the frequency, sensitivity, specificity, and clinical relevance of novel translocations.

The FISH assays used for the detection of the ETV6-NTRK3 fusion gene showed 80% sensitivity (four fusion-positive SBCs, one fusion-negative SBC) and 100% specificity (no false-positive results in 201 non-SBC breast cancers tested on TMA). This finding is in agreement with that of our previous study (Tognon et al., 2002). We conclude...
that the ETV6-NTRK3 gene fusion FISH assay is a useful ancillary technique for the diagnosis of SBC.

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REFERENCES


