Expression of the *ETV6-NTRK3* gene fusion as a primary event in human secretory breast carcinoma

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Summary

We report that human secretory breast carcinoma (SBC), a rare subtype of infiltrating ductal carcinoma, expresses the *ETV6-NTRK3* gene fusion previously cloned in pediatric mesenchymal cancers. This gene fusion encodes a chimeric tyrosine kinase with potent transforming activity in fibroblasts. *ETV6-NTRK3* expression was confirmed in 12 (92%) of 13 SBC cases, but not in other ductal carcinomas. Retroviral transfer of ETV6-NTRK3 (EN) into murine mammary epithelial cells resulted in transformed cells that readily formed tumors in nude mice. Phenotypically, tumors produced glands and expressed epithelial antigens, confirming that EN transformation is compatible with epithelial differentiation. This represents a recurrent chromosomal rearrangement and expression of a dominantly acting oncogene as a primary event in human breast carcinoma.

Introduction

Among human solid tumors, sporadic breast cancer is similar to the vast majority of epithelial malignancies in that it appears to have a complex genetic etiology. The numerous rearrangements and gains and losses of chromosomal material observed cytogentically in tumor cells have precluded the discovery of primary genetic lesions underlying breast cancer development (Dickson and Lippman, 2001). This is in contrast to bone and soft tissue sarcomas of children and young adults, which often show recurrent chromosomal translocations leading to expression of gene fusions thought to be causally related to oncogenesis (Ladanyi and Bridge, 2000). However, the occurrence of such translocations as the sole cytogenetic alteration in epithelial tumors is extremely rare.

We previously cloned the translocation breakpoints of the t(12;15)(p13;q25) associated with congenital fibrosarcoma (CFS), a pediatric spindle cell malignancy of the soft tissues (Knezevich et al., 1998b). This rearrangement generates a gene fusion encoding the sterile α motif (SAM) dimerization domain of the ETV6 (TEL) transcription factor linked to the protein tyrosine kinase (PTK) domain of the neurotrophin-3 receptor NTRK3 (TRKC) (Knezevich et al., 1998b). The resulting ETV6-NTRK3 (EN) protein functions as a chimeric PTK with potent transforming activity (Wai et al., 2000; Liu et al., 2000). EN expression leads to constitutive activation of two major effector pathways of wild-type NTRK3, namely the Ras-MAP kinase (MAPK) mitogenic pathway and the phosphatidyl inositol-3-kinase (PI3K)-AKT pathway mediating cell survival, and both are required for EN transformation (Wai et al., 2000; Tognon et al., 2001). *ETV6-NTRK3* fusion transcripts have also been identified in a related pediatric tumor, cellular mesoblastic nephroma (CMN) (Kneze-

SIGNIFICANCE

Mechanisms of oncogenesis in sporadic breast cancer remain for the most part obscure. This is due in large part to the marked cytogenetic complexity seen in most breast cancers, precluding investigators from readily identifying primary genetic events that may be causative in breast cell transformation. Our findings, although limited to a rare but distinct subset of breast cancers, describe a potentially causative genetic event in sporadic human breast carcinoma and establish ETV6-NTRK3 as a dominantly acting oncogene in this disease. Therefore, the transformation of breast epithelial cells by the ETV6-NTRK3 oncoprotein may provide an extremely useful model to study pathways underlying the initiation and progression of human breast cancer, and how those pathways may be targeted therapeutically.

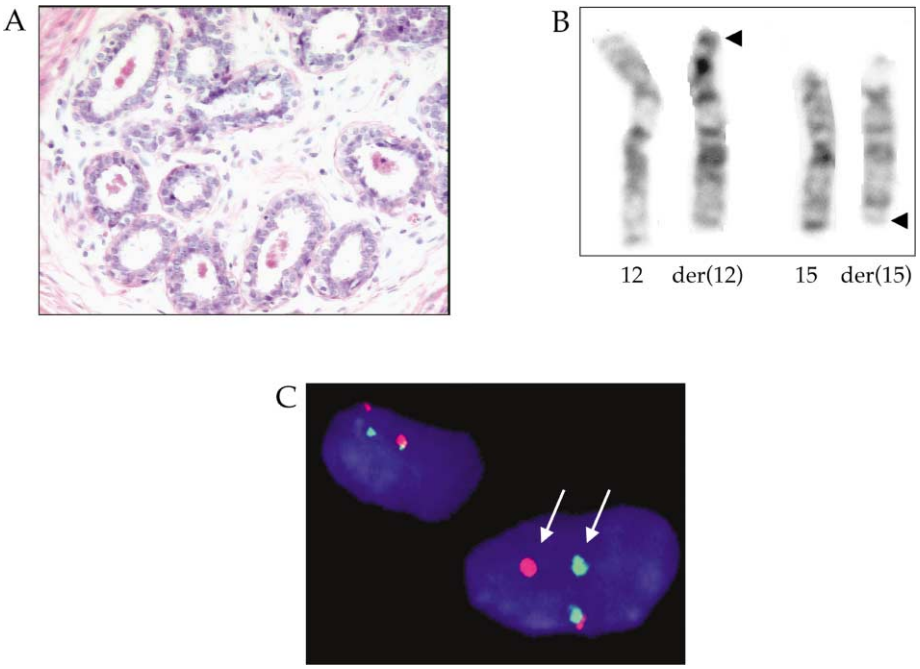

Figure 1. Histologic and cytogenetic findings in the index secretory breast carcinoma case

A: Histology of index secretory breast carcinoma (SBC) case demonstrating well-differentiated but invasive glands containing eosinophilic secretions.

B: Partial karyogram demonstrating the t(12;15)(p13;q25) in secretory breast carcinoma occurring in a 6-year-old female. Arrowheads show breakpoints at derivative 12p13 and derivative 15q25.

C: Dual color FISH using *ETV6* exon 1-containing cosmid 179A6 (green) and *ETV6* exon 8-containing cosmid 148B6 (Knezevich et al., 1998b). Arrows show separate signals indicating disruption of the *ETV6* gene.

vich et al., 1998a; Rubin et al., 1998), and in a case report of adult acute myeloid leukemia (AML) (Eguchi et al., 1999). Therefore, this gene fusion is unique in being expressed in both mesenchymal and hematopoietic lineages.

The possibility that *ETV6-NTRK3* fusion transcripts are expressed in human breast cancer was suggested to us by cytogenetic analysis of an invasive breast adenocarcinoma diagnosed in a 6-year-old female. This revealed a nonconstitutional t(12;15)(p12;q26.1) as the only karyotypic abnormality in tumor cells. The histopathologic features of the tumor were those of a secretory breast carcinoma (SBC). This is a rare but distinct subtype of infiltrating ductal carcinoma (IDC) that was originally described in children and adolescents, but is now known to occur with equal incidence in adults (Page et al., 1987; Oberman, 1980). Although recurrences and nodal metastases can occur in SBC, the prognosis was initially thought to be excellent compared to typical IDC, with up to a 100% five-year survival rate (Tavassoli and Norris, 1980; Rosen and Cranor, 1991). However, more recent studies have suggested that the favorable outcome is age-related, and that in older patients the prognosis is similar to IDC (Maitra et al., 1999). In this report we demonstrate that SBC is characterized by expression of the *ETV6-NTRK3* gene fusion, and that ETV6-NTRK3 can transform mammary epithelial cells. This fusion oncoprotein is therefore causally related to transformation across all germ layers, a finding which has potential implications for how we view the specificity of chromosomal translocations in human tumors.

Results

Identification of the t(12;15) translocation and *ETV6-NTRK3* gene fusion in a human secretory breast carcinoma case

The index case was a 6-year-old female who presented with a unilateral breast mass. Pathology revealed an invasive breast adenocarcinoma with the histopathologic features of a secretory breast carcinoma (SBC) (Figure 1A). SBC is characterized by an infiltrating pattern of neoplastic epithelial cells forming well-differentiated glandular lumens filled with eosinophilic PAS-positive secretions (Rosen and Cranor, 1991). Karyotypic analysis revealed a t(12;15)(p12;q26.1) translocation as the only cytogenetic abnormality in tumor cells (Figure 1B). This translocation was not present constitutionally. The karyotype prompted us to perform fluorescence *in situ* hybridization (FISH) and reverse transcriptase PCR (RT-PCR) on frozen tumor tissue from the case to screen for the *ETV6-NTRK3* gene fusion. As shown in Figure 1C, dual color FISH using cosmid probes from exon 1 and exon 8 of *ETV6* demonstrated rearrangement of the *ETV6* locus. Moreover, RT-PCR revealed an identical 731 bp fusion transcript as previously described for CFS and CMN (Knezevich et al., 1998a, 1998b) (Figure 2A). This was confirmed by sequencing of the amplification products, which demonstrated the identical fusion point between *ETV6* nucleotide 1033 and *NTRK3* nucleotide 1601 as previously shown for sarcoma-associated fusions (Knezevich et al., 1998b) (Figure 2B). This differs from the *ETV6-NTRK3* gene fusion reported in a case of acute myeloid leukemia, in which *ETV6* exon 5 was not present in the fusion (Eguchi et al., 1999). Furthermore, we generated RT-PCR products from the SBC case using the TEL 114 primer (located 5′ of the *ETV6* SAM sequence) in combination with the TRKC2 primer located within the *NTRK3* PTK sequence (Knezevich et al., 1998b), as well as using the TEL 541 primer from *ETV6* exon 5 (Knezevich et al., 1998b) in conjunction with the Trk1 primer (Wai et al., 2000) from exon 18 of *NTRK3* located 3′ of the PTK sequence (Ichaso et al., 1998) (see Figure 2B). These products were confirmed to contain identical sequences to those of CFS fusion transcripts and demonstrated that the entire SAM and PTK domains were included in the SBC transcripts (see GenBank accession #AF041811). We also found that, similar to CFS gene fusions, SBC transcripts did not contain *NTRK3* exon 16, which encodes a 42 bp insert within the PTK sequences (Ichaso et al., 1998). The presence of these naturally occurring inserts

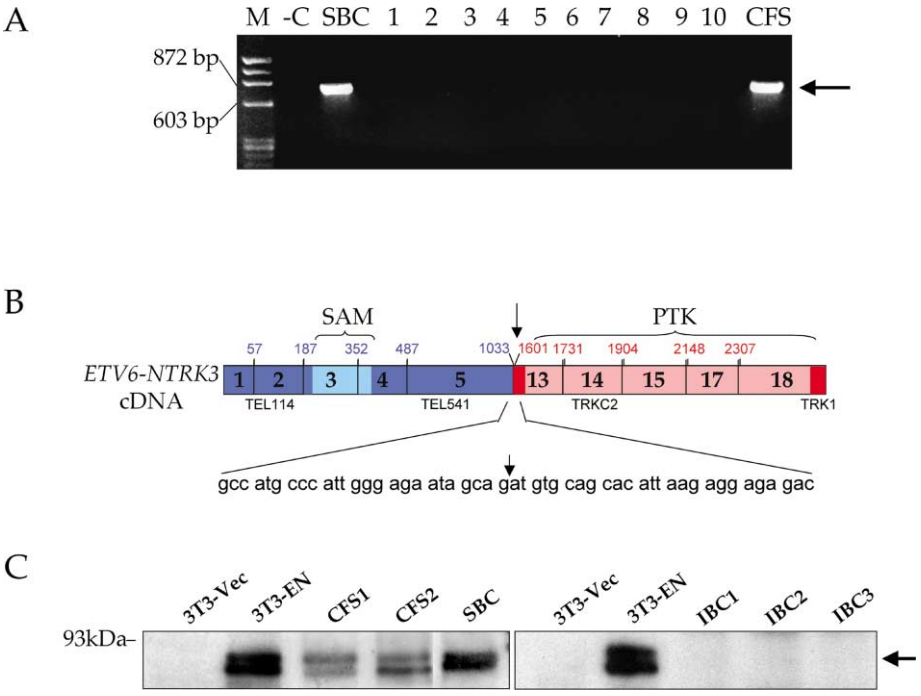

Figure 2. Characterization of the *ETV6-NTRK3* gene fusion and encoded protein in the index secretory breast carcinoma case

A: Reverse transcriptase-PCR of *ETV6-NTRK3* fusion transcripts using total RNA isolated from frozen tumor tissue of the index secretory breast carcinoma case (SBC) and a congenital fibrosarcoma control (CFS), but not from 10 infiltrating ductal carcinoma cases (lanes 1–10). M, markers; –C, negative control.

B: Schematic diagram showing the structure of the *ETV6-NTRK3* chimeric cDNA in secretory breast carcinoma. Exons 1–5 of ETV6 (blue boxes) are fused in frame with exons 13–15 and 17–18 of NTRK3 (red boxes). The lighter shade of blue indicates the region encoding the ETV6 sterile-α-motif (SAM) domain, while the lighter shade of red indicates the region encoding the NTRK3 protein tyrosine kinase (PTK) domain. Numbers above the exons indicate the last nucleotide of each exon. The fusion point is between *ETV6* nucleotide 1033 and *NTRK3* nucleotide 1601 (indicated by the vertical arrow) which is identical to that observed in congenital fibrosarcoma. The positions of the TEL114 and TEL541 forward primers and the TRKC2 and TRK1 reverse primers used to characterize *ETV6-NTRK3* fusion transcripts are shown under the exons (see text). An expanded view of the *ETV6-NTRK3* breakpoint sequence in the index secretory breast carcinoma case is shown below the cDNA schematic. This was derived by sequencing of RT-PCR products using primers TEL-541 and TRKC2. Identical sequences were observed in multiple clones from three separate experiments. The vertical arrow shows the fusion point.

C: Immunoprecipitation of the ETV6-NTRK3 protein using α-NTRK3 antibodies followed by Western analysis with α-phosphotyrosine antibodies. The left panel demonstrates the characteristic tyrosine phosphorylated 68/73 kDa ETV6-NTRK3 doublet in the index secretory breast carcinoma case (SBC), two CFS primary tumors (CFS1 and 2), and in NIH3T3 cells expressing ETV6-NTRK3 (3T3-EN), but not in the NIH3T3 vector control (3T3-Vec). The right panel demonstrates the doublet only in the 3T3-EN control cells but not in lysates from three invasive breast carcinoma cases (IBC1-3) nor in 3T3-Vec cells. The 68/73 kDa ETV6-NTRK3 doublet is indicated by the arrow.

is known to decrease NTRK3 PTK activity (reviewed in Barbacid, 1994).

To show that the ETV6-NTRK3 protein was expressed in tumor tissue, we performed immunoprecipitation of whole cell lysates from the primary tumor using α-NTRK3 antibodies followed by Western blotting with α-phosphotyrosine antibodies as described (Wai et al., 2000). This revealed expression of the characteristic tyrosine phosphorylated 68/73 kDa doublet of EN (Figure 2C, left panel), which was confirmed by reprobing of the blot with α-NTRK3 and α-ETV6 antibodies (data not shown). The tyrosine phosphorylated doublet was not detected in three control primary invasive ductal carcinomas that were negative for the *ETV6-NTRK3* gene fusion by RT-PCR (Figure 2C, right panel).

Analysis of *ETV6-NTRK3* transcript expression in a series of secretory breast carcinomas

To test whether *ETV6-NTRK3* expression represents a generalized finding in SBC, we accrued 12 additional breast cancer cases in which SBC was the predominant or only histologic component (for clinical features see Table 1). The diagnoses were independently confirmed by three of the authors (DH, CP, and PHBS). Only formalin-fixed paraffin-embedded (FFPE) tumor blocks were available for each case. We therefore used an RT-PCR assay adapted to FFPE tissue which detects a 110 bp fragment (Bourgeois et al., 2000). We detected *ETV6-NTRK3* fusion transcripts in 11 of the 12 cases, as well as in FFPE blocks from the index case (Figure 3A and Table 1). We successfully cloned PCR products from four of these additional positive cases, and sequence analysis confirmed that all four had breakpoint sequences that were identical to each other and to a control CFS case, with fusion points between *ETV6* nucleotide 1033 and *NTRK3* nucleotide 1601 (data not shown). To test whether this gene fusion is specific for SBC, we also screened a series of banked frozen specimens from 50 cases of typical IDC as well as FFPE blocks from 5 of these cases (cases 14–18 of Table 1). All were fusion negative except for one case (case 14 of Table 1), in which, unexpectedly, fusion transcripts could be demonstrated in both the frozen specimen and in the corresponding FFPE block from this case (Figure 3A).

To confirm these findings, we next performed dual color FISH analysis on nuclei isolated from FFPE blocks using yeast artificial chromosomes (YACs) containing the *ETV6* and *NTRK3* loci, respectively. Nine of the 13 SBC cases (including the index case) had adequately preserved nuclei for FISH analysis, as did each of the five IDC FFBE blocks. The nine SBC cases with evaluable nuclei were all positive by FISH for the *ETV6-NTRK3* gene fusion (see Figure 3B and Table 1). Moreover, the IDC case that was positive by RT-PCR (case 13) was also positive by FISH while the other FFBE blocks were negative (Table 1). Further review of pathologic material from case 14 revealed several areas of well-differentiated glands containing eosinophilic secretions (data not shown). Therefore this case may have originated as an SBC, but underwent further clonal evolution to assume a less differentiated phenotype. These findings strongly indicate that the *ETV6-NTRK3* gene fusion is a nonrandom rearrangement in SBC.

Table 1. Breast carcinomas tested for t(12;15)-associated *ETV6-NTRK3* fusion transcripts by reverse transcriptase-PCR (RT-PCR) and fluorescence in situ hybridization (FISH)

Case	Sex	Age at Dx (yrs)	Diagnosis	RT-PCR	FISH
1	f	6	secretory carcinoma	pos	pos
2	f	19	secretory carcinoma	pos	NI
3	m	75	gynecomastia with secretory carcinoma	neg	NI
4	f	57	secretory carcinoma	pos	NI
5	f	49	secretory carcinoma	pos	NI
6	f	59	secretory carcinoma	pos	pos
7	f	39	mixed ductal and secretory carcinoma	pos	pos
8	f	64	secretory carcinoma	pos	pos
9	f	76	secretory carcinoma	pos	pos
10	f	63	secretory carcinoma	pos	pos
11	f	63	secretory carcinoma	pos	pos
12	f	62	secretory carcinoma	pos	pos
13	f	42	secretory carcinoma	pos	pos
14	f	63	ductal carcinoma	pos	pos
15	m	16	ductal carcinoma	neg	neg
16	f	84	ductal carcinoma	neg	neg
17	f	59	ductal carcinoma	neg	neg
18	f	56	ductal carcinoma	neg	neg

Abbreviations: Dx, diagnosis; f, female; m, male; pos, positive; neg, negative; NI, not informative.

ETV6-NTRK3 transforms Eph4 and Scg6 breast epithelial cells

To demonstrate that ETV6-NTRK3 has transformation activity in breast epithelial cells, we used previously described retroviral gene transfer methods (Wai et al., 2000) to express EN constructs versus vector alone in clonal populations of murine Eph4 and Scg6 cells. These are immortalized nontransformed epithelial cell lines derived from normal mouse mammary epithelium (Roskelley et al., 2000; Somasiri et al., 2001). We chose these lines because they represent opposite ends of the spectrum of mammary epithelial differentiation, with Scg6 cells showing stable myoepithelial (more mesenchymal) features and Eph4 cells showing a stable epithelial phenotype in culture. After infection with ETV6-NTRK3 constructs, both cell lines were found to express ETV6-NTRK3 at levels that were equivalent to those observed in EN-transformed NIH3T3 cells (Figure 4A). Morphology of Eph4-EN and Scg6-EN cells in monolayer cultures suggested a transformed phenotype compared with vector alone cells (data not shown). To more rigorously demonstrate that EN had transforming ability in these cells, EN-expressing and vector alone cells were simultaneously injected subcutaneously into the right or left flanks, respectively, of nude mice. Tumor formation was then monitored for up to six weeks in comparison with EN-expressing NIH3T3 cells. Eph4 and Scg6 cells containing vector alone showed no tumor formation whatsoever (Figure 4B). In contrast, both cell lines formed obvious tumors when engineered to express EN. All Eph4-EN and Scg6-EN injection sites showed tumor formation, although tumor sizes were variable (Figure 4B). Interestingly, tumors derived from the two breast epithelial cell lines consistently grew more slowly compared with EN-expressing NIH3T3 cells, although they eventually attained the same sizes and weights (Figure 4C). These findings confirm that EN has transformation activity in breast epithelial cells, and strongly implicate EN as being causally related to oncogenesis in human SBC.

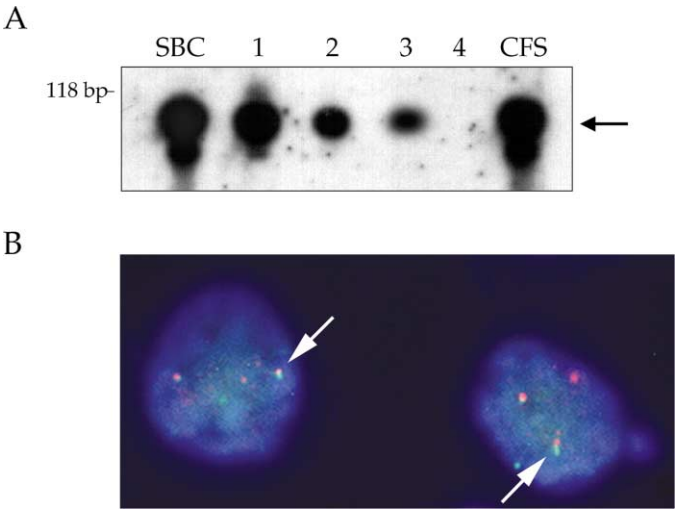

Figure 3. Detection of the *ETV6-NTRK3* gene fusion in a series of secretory breast carcinomas

A: Reverse transcriptase-PCR of *ETV6-NTRK3* fusion transcripts (arrow) using total RNA isolated from formalin-fixed paraffin-embedded tumor blocks of the index secretory breast carcinoma case (SBC) as well as in cases 2, 3, and 14 from Table 1 (lanes 1–3, respectively), but not in case 15 (lane 4). CFS, congenital fibrosarcoma control.

B: Dual-colored FISH of CFS case 2 metaphase using the 12p13 breakpoint-spanning YAC, 817_H_1 (green), and the 15q25 breakpoint-spanning YAC, 802_B_4 (red) (Knezevich et al., 1998b). Representative fusion signals are shown by the arrows.

Pathology of tumors derived from ETV6-NTRK3 expressing Eph4 and Scp6 cells

We next assessed histopathologic sections from Scg6 and Eph4 derived tumors expressing EN. All Scg6-EN tumors had a mesenchymal phenotype that was indistinguishable from the fibrosarcoma appearance of EN-expressing NIH3T3 tumors (see Fig-

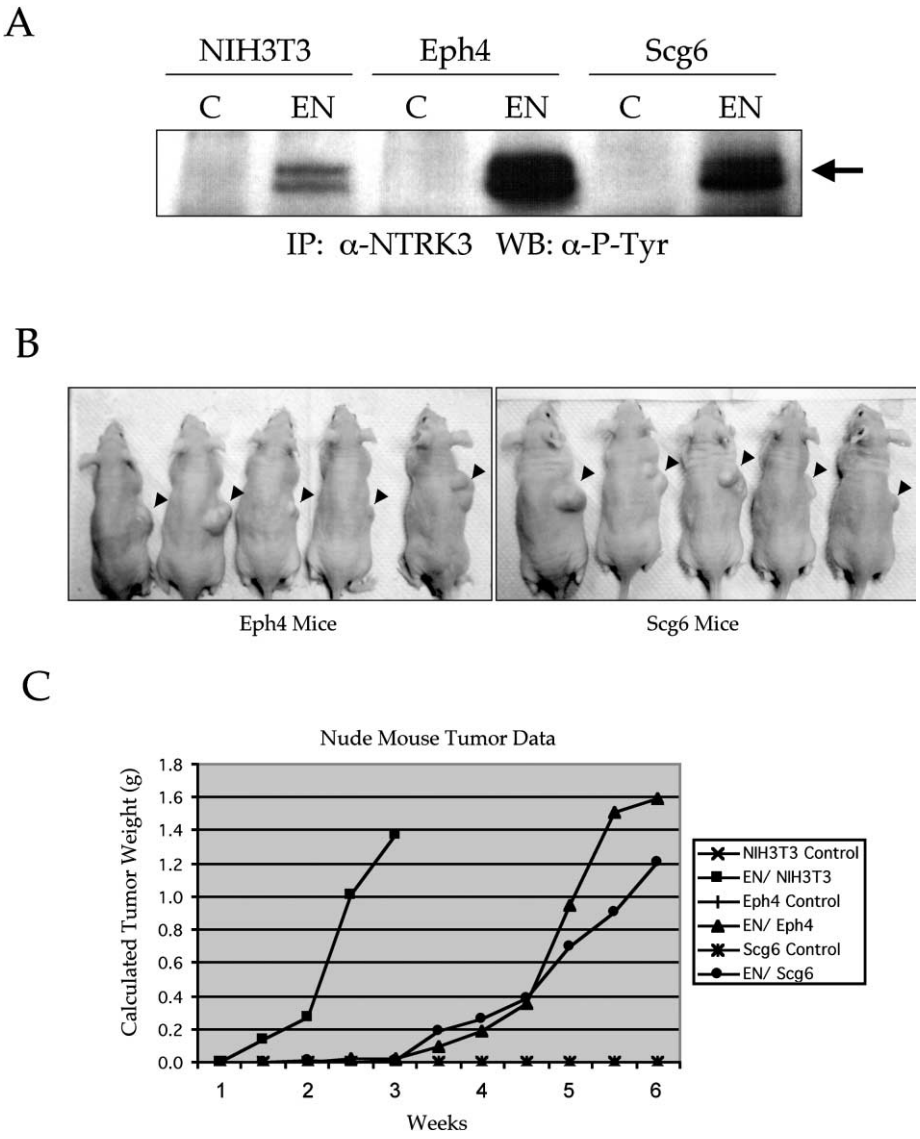

Figure 4. Transformation of murine breast epithelial cells by the ETV6-NTRK3 chimeric oncoprotein

A: Expression of ETV6-NTRK3 in Eph4 and Scg6 breast epithelial cells. NIH3T3 fibroblasts and Eph4 or Scg6 breast epithelial cells were infected with retroviruses containing control vector alone (C) or ETV6-NTRK3 (EN) and were grown to near confluence. Whole cell lysates (500 μg) were then collected and subjected to immunoprecipitation with α-NTRK3 antibodies. Immunoprecipitates were separated by SDS-PAGE and subjected to 4G10 anti-phosphotyrosine immunoblotting. A tyrosine phosphorylated doublet at 68 and 73 kDa is seen in lysates from EN infected cells.

B: Tumor formation after injection of Eph4 and Scg6 cells expressing ETV6-NTRK3. Vector control Eph4 or Scg6 cells were injected subcutaneously into the left flanks of nude mice, while Eph4 or Scg6 cells expressing ETV6-NTRK3 were simultaneously injected into the right flanks of the same mice. As indicated by the arrowheads, tumors formed only on the right flanks of these mice.

C: Growth rates for nude mouse tumors after subcutaneous cell injections. There was no tumor growth for NIH3T3, Eph4, and Scg6 control cells expressing vector alone. In contrast, the same cell lines expressing ETV6-NTRK3 (EN-NIH3T3, EN-Eph4, and EN-Scg6, respectively) all formed identifiable tumors, which were monitored for size over a six week period. The growth curves shown are representative results for the largest tumors observed for each cell line.

ures 5A and 5B). There was no gland formation or other evidence of epithelial differentiation. As shown in Figures 5D and 5E, NIH3T3-EN and Scg6-EN cells were negative by immunohistochemistry for cytokeratin or other epithelial markers, but were positive for the mesenchymal intermediate filament, vimentin (Figures 5G and 5H). In contrast, Eph4-EN derived tumors showed obvious gland formation and sheets of epithelioid cells (Figure 5C). Gland cells were strongly positive for cytokeratin (Figure 5F) and epithelial membrane antigen (data not shown) but negative for vimentin (Figure 5I). Adjacent to the epithelial clusters were bands of vimentin-positive, cytokeratin-negative cells with a similar mesenchymal appearance as the Scg6-EN tumors. There was an approximately equal contribution of the epithelial and mesenchymal neoplastic cells to the Eph4-EN tumors. Immunohistochemical staining of sections using α-NTRK3 antibodies demonstrated EN expression in the NIH3T3-EN and Scg6-EN cells (Figure 5J and 5K), and in both the glandular and mesenchymal elements of the Eph4-EN derived tumors (Figure 5L). These data show that EN-expressing tumor cells maintained the ability to undergo epithelial differentiation, which is consistent with our premise that EN is a transforming oncoprotein not only in mesenchymal cells but also in breast epithelial cells.

Discussion

In this report we show that human secretory breast carcinoma is characterized by expression of the t(12;15)-associated *ETV6-NTRK3* gene fusion. Moreover, we demonstrate that the ETV6-NTRK3 chimeric product can transform normal mouse mammary epithelial cells, strongly implicating this oncoprotein as being causally related to the development of SBC. SBC therefore joins a very select group of epithelial malignancies, namely subtypes of thyroid carcinomas (Pierotti, 2001), renal carcinomas (Cooper and Shipley, 2002), and aggressive sinonasal carcinoma (French et al., 2001), for which recurrent gene fusions have been described. *ETV6-NTRK3* is unique among these gene fusions in that it is expressed in multiple tumor types including

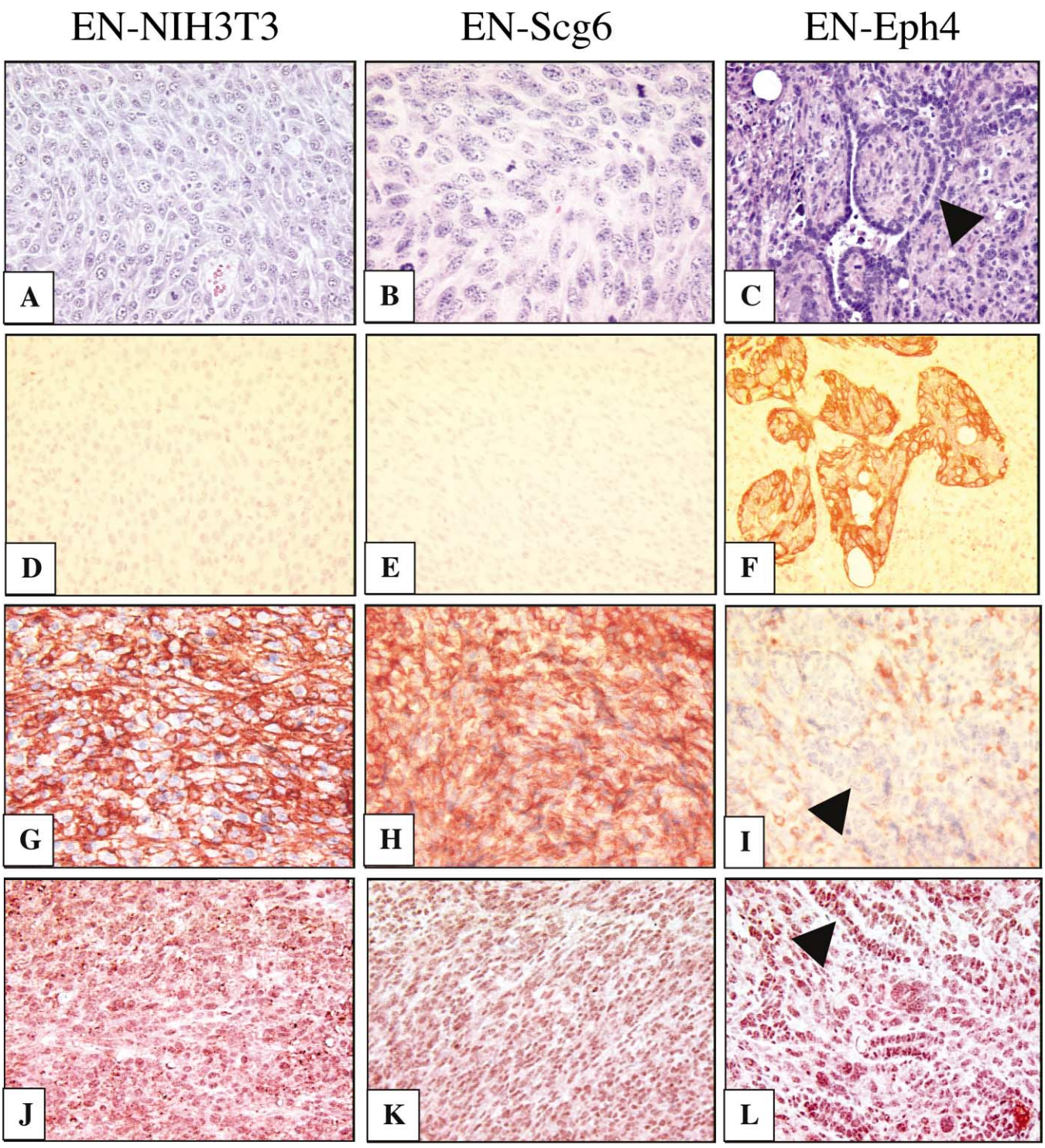

Figure 5. Histology of tumors formed after injection of ETV6-NTRK3 expressing NIH3T3 cells (EN-NIH3T3), Scg6 cells (EN-Scg6), or Eph4 cells (EN-Eph4) into nude mice

No tumors formed after injection of vector alone control cells. **A–C:** Photomicrographs of hematoxylin & eosin stained tumor sections. Formation of glandular and papillary epithelial structures (e.g., arrowhead in **C**) was observed only in EN-Eph4 tumors. **D–L:** Immunohistochemical staining of sections from EN-NIH3T3, EN-Scg6, or EN-Eph4 tumors using antibodies directed against the epithelial intermediate filament cytokeratin (**D–F**), the mesenchymal intermediate filament vimentin (**G–I**), and NTRK3 as a marker of ETV6-NTRK3 expression (**J** and **K**). Arrowheads in the EN-Eph4 panels indicate positive immunostaining of glandular structures for cytokeratin (**F**), lack of immunostaining of glandular structures for vimentin (**I**), and positive immunostaining in both mesenchymal and glandular elements for ETV6-NTRK3 (**L**).

those derived from mesenchymal (Knezevich et al., 1998a, 1998b; Rubin et al., 1998), hematopoietic (Eguchi et al., 1999), and now epithelial cells.

The identification of an *ETV6-NTRK3* gene fusion in SBC describes a recurrent chromosomal rearrangement and expression of a dominantly acting oncogene as a primary event in breast carcinoma. The chromosomal instability observed in the vast majority of epithelial malignancies, leading to numerous rearrangements, gains, and losses of chromosomal material (Dickson and Lippman, 2001), has precluded the discovery of primary genetic events underlying the development of epithelial tumors. If expression of *ETV6-NTRK3* is indeed a causal event in secretory breast carcinoma, then transformation of breast epithelial cells by the EN oncoprotein may provide an extremely useful model to study pathways leading both to the initiation and progression of breast cancer. In fact, there are several parallels between the signaling alterations described for EN fibroblast transformation and those observed in breast cancer. We previously reported that EN is a potent transforming protein in NIH3T3 cells and that this activity requires a functional NTRK3 PTK domain (Wai et al., 2000). Moreover, EN transformation leads to marked induction of cyclin D1 and is dependent on two major effector pathways of wild-type NTRK3, the Ras-MAPK pathway and the PI3K-AKT pathway (Wai et al., 2000; Tognon et al., 2001). Amplification and overexpression of the cyclin D1 (*CCND1*) gene is a well-known progression-related alteration in breast cancer (Buckley et al., 1993; Wang et al., 1994; Sicinski et al., 1995), being present in approximately one-third of invasive cases (Zukerberg et al., 1995). Moreover, the Ras-MAPK and PI3K-AKT pathways are receiving increasing attention as potential therapeutic targets in breast cancer (reviewed in Malaney and Daly, 2001, and Mills et al., 2001, respectively). Another pathway suspected to play a key growth regulatory role in breast cancer is the insulin-like growth factor 1 receptor (IGFRI) cascade (reviewed in Zhang and Yee, 2000). Interestingly, we recently found that expression of functional IGFRI is essential for EN-mediated transformation, as EN fails to transform IGFRI null fibroblasts (Morrison et al., 2002). EN transformation may provide a unique model to study how these and other pathways contribute to breast oncogenesis. It will also be important to determine whether activation of the NTRK receptor PTK family by alternative mechanisms is a more general phenomenon in human breast cancer. In fact, recent reports document NTRK expression in malignant breast epithelial cells (Descamps et al., 1998, 2001b, 2001a).

Tumors derived from EN-expressing Eph4 cells showed epithelial differentiation with cytokeratin- and EMA-positive gland formation. Therefore not only is EN capable of transforming cells with epithelial developmental potential, but it does not block epithelial differentiation, in keeping with the well-differentiated histology of human SBC. However, the fact that EN expression is associated with tumors derived from multiple lineages calls into question the concept that gene fusions are specific for given tumor types (Rabbitts, 1994). Two possible mechanisms have been proposed to explain the strong association between acquired chromosomal translocations and specific tumor types that has been documented in hematopoietic malignancies and sarcomas (Barr, 1998). The first proposes that the lineage of a cell imposes constraints on whether or not a particular chimeric oncoprotein will be expressed or have pathologic consequences in that cell type. For example, if the functional promoter of a given chimeric gene is not active in a particular cell type, then the product of that gene will not be expressed. Similarly, if a chimeric oncoprotein is generally not tolerated in cells, e.g., if it induces apoptosis, then it will only be expressed in cell types with mechanisms for circumventing oncogene-induced apoptotic pathways. Evidence for the latter comes from recent studies of the Ewing tumor-associated EWS-FLI1 chimeric transcription factor. Expression in primary human fibroblasts activated a p53-dependent growth arrest pathway and down-regulated numerous cell cycle progression related genes (Lessnick et al., 2002). This suggests that cells transformed by EWS-FLI1 must coactivate pathways that specifically abrogate EWS-FLI1-induced growth arrest in order for mitogenesis to occur and for tumors to form. The second explanation for tumor specificity of fusion genes proposes that the chimeric oncoprotein itself can induce differentiation, giving rise to the observed phenotype. Many gene fusions in hematopoietic malignancies and sarcomas encode chimeric transcription factors (Sorensen and Triche, 1996). Therefore these proteins may transcriptionally activate specific differentiation programs. EWS-FLI1 expression in NIH3T3 cells was associated with a small round cell morphology and limited neural differentiation in nude mouse tumors (Teitell et al., 1999), and a myogenic transcription program was induced in NIH3T3 cells expressing the alveolar rhabdomyosarcoma-associated PAX3-FKHR protein (Khan et al., 1999).

We favor the view that some chimeric oncoproteins such as EN may activate transformation pathways that function independently of lineage constraints and do not directly affect differentiation programs. These oncoproteins may modulate common signal transduction cascades present in diverse cell types that potentiate downstream proliferative effects such as transit through the cell cycle. In such a scenario it may be the ability of the cell to tolerate the potentially toxic consequences of fusion protein expression, such as by activating complementary cell survival pathways, which determines whether or not tumor formation occurs. In this context, the observed morphology of neoplastic cells results from developmental programs already present in tumor precursor cells. Therefore the perceived specificity of gene fusions may be more a function of the available complement of secondary pathways or the tendency for required additional mutations to occur than of the intrinsic differentiation program available in the precursor cell.

Experimental procedures

Patient information

The clinical features of the 13 SBC cases assessed in this study are listed in Table 1. The cases were accrued from Pathology departments at four of the authors' institutions (Children's and Women's Health Centre of British Columbia, Hospital for Sick Children, Instituto de Patologia e Imunologia Molecular da Universidade do Porto, and Gerhard-Domagk-Institute of Pathology, Westfaelische-Wilhelms University) and from Burnaby General Hospital, Burnaby, Canada. The age range was 6 to 76 years, and there was a 12:1 female to male ratio. Assessment of the pathologic records and independent review of slides from each case confirmed the secretory component as being the predominant or exclusive morphology present in the tumors, other than in cases 3 and 7. In case 3 the secretory carcinoma component was present within a background of gynecomastia, while in case 7 the secretory component was admixed approximately equally with non-SBC infiltrating ductal carcinoma. All blocks used for molecular analysis were screened for the presence of at least 50% viable tumor tissue. Table 1 also includes clinical information on five infiltrating ductal carcinoma cases from which formalin-fixed paraffin-embedded blocks were used as controls for molecular analysis.

Cytogenetic analysis

Tumor tissue for cytogenetic analysis was collected from the index case at the time of surgery. Cytogenetic techniques used for chromosome analysis have been described previously (Sandberg and Bridge, 1994). The samples were mechanically and enzymatically disaggregated and cultured in RPMI 1640 medium supplemented with 20% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine for several days. Six hours before harvesting, cells were exposed to Colcemid (0.02 μg/ml). After a brief incubation in hypotonic solution, the preparations were fixed with methanol-glacial acetic acid (3:1). Metaphase cells were banded with Wright stain or with Giemsa trypsin. A minimum of 20 metaphases were analyzed for each specimen. Karyotypes were in accordance with the International System for Human Cytogenetic Nomenclature (1995).

RT-PCR analysis of breast tumor specimens

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to screen for *ETV6-NTRK3* fusion transcripts in frozen tissue samples from the index case and an additional 50 control cases of infiltrating ductal carcinoma using previously described methods (Knezevich et al., 1998b). Total RNA was used as starting material and was isolated using standard procedures. The TEL541 forward primer (5′-CCTCCCACCATTGAACTGTT-3′) and the TRKC2 reverse primer (5′-CCGCACACTCCATAGAACTTGAC-3′) were used for PCR. Amplified products were visualized by electrophoresis using 2% agarose gels stained with ethidium bromide. *ETV6-NTRK3* amplification products were confirmed by blotting the PCR fragments onto Hybond-N nylon filters followed by hybridization with an oligonucleotide probe as described (Bourgeois et al., 2000), and by sequencing of the PCR products. The presence of amplifiable RNA in all samples was confirmed by RT-PCR using control primer sets.

RT-PCR of *ETV6-NTRK3* fusion transcripts in formalin-fixed paraffin-embedded sections was based on previously described methods (Bourgeois et al., 2000). Briefly, RNA was isolated from three 30 μm paraffin sections and used directly in a one-step RT-PCR reaction using the Biosciences Titanium One Step RT-PCR kit (Clontech). First, the RNA was checked for integrity using primer pairs for the phosphoglycerate kinase (PGK) gene, as described previously (Bourgeois et al., 2000). The RNA was then subjected to RT-PCR using sense TEL971 (5′-ACCACATCATGGTCTCTCTGTCTCCC-3′) and antisense TRKC1059 (5′-CAGTTCTCGCTTCAGCACGATG-3′) primers (Bourgeois et al., 2000). PCR conditions were as reported previously, with the assay detecting a 110 bp PCR fragment. Products were confirmed as *ETV6-NTRK3* transcripts by oligonucleotide probing as described above.

Fluorescence in situ hybridization (FISH)

Two FISH assays were used to detect the t(12;15) translocation in SBC. Slides containing interphase cell spreads of the index case were prepared from frozen tumor tissue and subjected to dual-color FISH as previously described (Knezevich et al., 1998b). Probes used included *ETV6* exon 1-containing cosmid 179A6 and *ETV6* exon 8-containing cosmid 148B6, which become separated in cells with the t(12;15) translocation (Knezevich et al., 1998b). For analysis of chromosome breakpoints in formalin-fixed paraffin-embedded tumor blocks, 5 μm sections were first deparaffinized and dehydrated in 100% ethanol using standard methods. Slides were boiled in 2xSSC for 30 s, washed, and then pretreated in 1M sodium thiocyanate for 30 min. Sections were then digested using 5 mg/ml pepsin in 0.2N HCL at 37°C for 20 min. This was followed by fixation in formaldehyde, dehydration with ethanol, and denaturation with 70% formamide/2xSSC (pH 7.0) at 90°C for 6 min. Dual-color FISH was performed using the 12p13 breakpoint-spanning YAC, 817_H_1 and the 15q25 breakpoint-spanning YAC, 802_B_4 (Knezevich et al., 1998b). Probes were labeled with Spectrum Green (817_H_1) and 0.2 Spectrum Red (802_B_4) by nick translation following standard protocols, and hybridized to denatured slides in a humid chamber at 37°C overnight. These probes become juxtaposed in cells with the t(12;15) translocation (Knezevich et al., 1998b). Slides were counterstained with DAPI and analyzed using a Zeiss Axioplan epifluorescent microscope equipped with a COHU-CCD camera.

Cell lines

NIH3T3 cells were obtained from ATCC and maintained at low confluence in Dulbecco's Modified Eagle Medium (DMEM; GIBCO-BRL) containing 10% calf serum (CS; GIBCO-BRL). The BOSC23 packaging cell line was obtained from Dr. Rob Kay (Terry Fox Laboratory, Vancouver, Canada) and grown in DMEM containing 10% fetal calf serum (FCS; GIBCO-BRL). The mouse mammary epithelial cells lines, Scg6 and Eph4, were obtained from Dr. Calvin Roskelley (Dept. of Anatomy, University of British Columbia, Vancouver, BC, Canada) and maintained in DMEM/F12 (Sigma) supplemented with 5% FBS (Hyclone), insulin (5 μg/ml; Sigma), and gentamicin (50 μg/ml, GIBCO-BRL).

Generation of retrovirally transduced cell lines

Retroviral vector plasmid DNAs (MSCV-puromycin vector or MSCV puromycin ETV6-NTRK3 [Wai et al., 2000]) were transfected into the BOSC23 ecotropic retroviral packaging cell line using calcium phosphate precipitation as described (Pear et al., 1993). Supplemental Gag/Pol (pGP1) and Env plasmids were used during the transfection procedure to increase viral titres. Retrovirus-containing supernatants were collected 48 hr after transfection and used to infect NIH3T3, Scg6, and Eph4 cells. Infected cells were selected for using 1.5 μg/ml puromyocin (Sigma) for 48 hr. Protein expression was determined by Western blotting as described (Wai et al., 2000).

Immunoprecipitation studies

Lysates were prepared from scrolls of frozen tumor samples embedded in OCT using a RIPA lysis buffer consisting of 20 mM Tris pH 7.4; 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4, plus freshly added protease inhibitors (Leupeptin [10 μg/ml], Apoprotinin [10 μg/ml], and PMSF [250 μM]). Lysates prepared from cell lines were prepared as described previously (Tognon et al., 2001). Protein quantification was performed using a DC protein assay kit (BioRad). Immunoprecipitation studies to detect ETV6-NTRK3 were performed on 500 μg of total cell lysate (TCL) using 5 μl of α-TrkC 14 antibodies (Santa Cruz) and 20 μl of Protein A sepharose (Amersham Pharmacia Biotech). Washed immunoprecipitates were electrophoresed using 10% SDS-polyacrylamide gels, and transferred to Immobilon-P (Millipore) PVDF prior to immunoblot analysis with 4G10 α-phosphotyrosine antibodies (Upstate). Proteins were visualized with ECL (Amersham) according to the manufacturer's protocols. Blots were stripped and reprobed with α-TrkC14 antibodies to verify the presence of ETV6-NTRK3.

Tumor growth in nude mice

Two million NIH3T3, Eph4, or Scg6 cells expressing either control MSCV vectors or *ETV6-NTRK3* constructs were injected subcutaneously into the left flanks (MSCV cell lines) or right flanks (*ETV6-NTRK3* expressing cell lines) of five nude male mice per cell line. Tumor injection sites were monitored three times weekly. Calliper measurements were taken when the tumors were large enough to assay. Tumors were excised and weighed after 20 days (for NIH3T3 cells) or 48 days (for Eph4 and Scg6 cell lines) and average tumor weights were calculated. Tumor growth over time was estimated using the following equation: tumor length × tumor width × tumor height × 0.5236.

Tumor histology

Sections from excised tumors were fixed in 10% formalin and embedded in paraffin using standard protocols. Sections were cut at 5 μm and stained with hematoxylin and eosin (H/E). Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded sections using a three-step streptavidin-biotin peroxidase method, with 3-amino-9-ethylcarbazole as the chromogen. Antibodies utilized included cytokeratin (Z0622 from Dako; 1:1000 dilution), epithelial membrane antigen (EMA; ID Laboratories; 1:50), carcinoembryonic antigen (CEA; Dako; 1:200), NTRK3 (TRKC14 from Santa Cruz; 1:40), and vimentin (Nova Castro; 1:100). Microwave antigen retrieval was performed using standards methods. Photomicrographs were taken with a Zeiss Axioplan 2 microscope equipped with Northern Eclipse version 5.0 software.

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