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Is acinic cell carcinoma a variant of secretory carcinoma? A FISH study using *ETV6* 'split apart' probes

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Aims: Acinic cell carcinomas (ACCs) and secretory carcinomas (SCs) of the breast are rare, low-grade malignancies that preferentially affect young female patients. Owing to the morphological and immunohistochemical similarities between these lesions, they have been proposed to be two morphological variants of the same entity. It has been demonstrated that SCs of the breast consistently harbour the t(12;15)*ETV6-NTRK3* translocation. The aim was to determine whether ACCs also harbour *ETV6* gene rearrangements and are thus variants of SCs.

Methods and results: Using the *ETV6* fluorescence *in situ* hybridization DNA Probe Split Signal (Dako), the presence of *ETV6* rearrangements in three SCs and six ACCs was investigated. Cases were considered as harbouring an *ETV6* gene rearrangement if >10% of nuclei displayed 'split apart signals' (i.e. red and green signals were separated by a distance greater than the size of two hybridization signals). Whereas the three SCs displayed *ETV6* split apart signals in >10% of the neoplastic cells, no ACC showed any definite evidence of *ETV6* gene rearrangement.

Conclusions: Based on the lack of *ETV6* rearrangements in ACCs, our results strongly support the concept that SCs and ACCs are distinct entities and should be recorded separately in breast cancer taxonomy schemes.

Keywords: acinic cell carcinoma, breast cancer, chromosomal translocation, classification, fluorescence *in situ* hybridization, molecular pathology

Abbreviations: ACC, acinic cell carcinoma; BAC, bacterial artificial chromosome; CCD, charge coupled device; DAPI, 4′-6-diamidino-2-phenylindole; ER, oestrogen receptor; FISH, fluorescence *in situ* hybridization; GCDFP, gross cystic disease fluid protein; PBS, phosphate-buffered saline; PR, progesterone receptor; SC, secretory carcinoma; SSC, NaCl and sodium citrate

Introduction

Acinic cell carcinoma (ACC) was first described in the breast by Roncaroli *et al.*,[1] in 1996, as one of the types of salivary gland-like tumours of the breast. Since the original description, only 11 additional cases have been reported.[2–10] ACC preferentially affects women, with a wide-range distribution of 35–80 years of age.

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Clinically, all tumours reported to date have presented as palpable nodules. Histologically, ACC is composed of cells arranged in a complex admixture of solid, microcystic and microglandular areas, with a histopathological appearance superficially reminiscent of microglandular adenosis.[4] These cells have round-to-ovoid nuclei containing a single, conspicuous nucleolus. Their cytoplasm is usually abundant, amphophilic-to-eosinophilic and granular.[1–10] The granules vary from bright eosinophilic, resembling those seen in Paneth cells, to amphophilic. Immunohistochemically, these tumours are characterized by the lack of oestrogen (ER) and progesterone (PR) receptor expression, lack of HER2 positivity and expression of markers usually seen in acinic cells of the salivary glands, including amylase, lysozyme and chymotrypsin.[1–10] In addition, positivity for epithelial membrane antigen and S100 protein is frequently found and focal expression of gross cystic disease fluid protein 15 (GCDFP15) is not uncommon.[1–10] ACCs are reported to have a rather indolent behaviour, with only two out of 11 cases presenting with lymph node metastasis, and only one case of ACC-related death reported to date.[1–10]

Secretory carcinoma (SC) of the breast, also known as juvenile carcinoma, is a low-grade malignancy first described by McDivitt and Stewart[11] in 1966. These tumours were first thought to be restricted to prepubertal patients, but more recent studies have demonstrated that only approximately one-third of SCs are diagnosed in children. In fact, the modal age of SCs seems to be in the third decade.[12] SCs usually present as mobile lumps in the peri-areolar region, but can be found in any part of the breast, and are more frequently found in female patients. Histologically, SCs comprise partially circumscribed nodules composed of cells arranged in solid, microcystic and tubular areas. Abundant pale pink or amphophilic, 'bubbly' secretion is found in tumour cells, glands and microcystic spaces. Neoplastic cells characteristically have low-grade nuclei, sometimes with vesicular chromatin and discrete nucleoli and cytoplasm that ranges from pale to eosinophilic to amphophilic. Cytoplasmic vacuolization is almost uniformly found in these tumours, and signet ring cells are commonly seen. Immunohistochemically, SCs are often negative for ER and PR, lack HER2 3+ overexpression and *HER2* gene amplification and are frequently positive for α-lactalbumin, S100 protein and polyclonal carcinoembryonic antigen.[13,14] The reported clinical behaviour of SC suggests that these tumours have a rather indolent course, being less aggressive than invasive ductal carcinomas of no special type and with a proclivity for late local recurrences and prolonged survival.[12,15–17] Lymph node spread is rarely seen[15–17] and even when present the prognosis of SC still appears good.[15–17]

It has recently been demonstrated that SCs harbour a recurrent balanced chromosomal translocation t(12;15)(p13;q25), which leads to a fusion gene between the *ETV6* gene from chromosome 12 and the *NTRK3* gene from chromosome 15.[18] The fusion product has been shown to have transformation activity in mouse mammary gland myoepithelial and epithelial cells, Eph4 and Scg6, respectively. Surprisingly, the morphological features and phenotypes of Eph4 and Scg6 xenografts were remarkably different and none of them were similar to those of secretory carcinomas. EN-Scg6 xenografts showed a sarcomatoid appearance, whereas EN-Eph4 xenografts were biphasic tumours.[18] Despite the fact that the xenograft models do not mimic the human disease, it has been shown that the t(12;15) translocation is seen in most if not all SCs.[14,18–21]

In a recent study, Hirokawa *et al.*[22] have reported on the clinicopathological and immunohistochemical findings of three cases of secretory carcinoma. Based on the similarities of the morphological patterns of their cases when compared with those of ACC, on the expression of salivary-type amylase, lysozyme, S100 protein and α_1-antitrypsin, and lack of GCDFP15 and ER,[22] on the fact that both ACC and SC are low-grade malignancies that preferentially affect young female patients, the authors hypothesized that these lines of evidence would 'indicate that they (ACC and SC) are identical lesions'.[22]

We hypothesised that if ACCs were variants of SCs, it would be reasonable to expect that ACCs would also harbour *ETV6* gene rearrangements and, in particular, the t(12;15)(p13;q25) chromosomal translocation, which has been consistently reported in SCs. In this study, we used a two-tiered approach to investigate the presence of the *ETV6/NTRK3* translocation in ACCs. We first investigated the presence of *ETV6* rearrangements in a series of SCs and ACCs of the breast using a commercially available *ETV6* 'split apart' probe. Subsequently, the presence of the t(12;15)(p13;q25) *ETV6/NTRK3* translocation was confirmed with translocation 'fusion probes' in cases harbouring *ETV6* 'split apart' signals.

Materials and methods

Six cases of pure ACC and three cases of SC of the breast were retrieved from the consultation files of three of the authors (J.S.R-F., V.E., I.O.E.). Haematoxylin and eosin-stained slides of the tumours were reviewed and the diagnoses of ACC and SC of the breast were confirmed based on the criteria proposed by Damiani *et al.*[4]

Sections (4 μm thick) from representative blocks were cut and mounted on polylysine-coated slides. Slides were anonymized before being sent to the Molecular Pathology Laboratory at The Breakthrough Breast Cancer Research Centre. Owing to the nature of the study, the demographics and clinical details of patients were not retrieved from the pathology reports. This retrospective study was approved by the Royal Marsden Hospital Research Ethics Committee and by the Nottingham Research Ethics Committee 2 under the title of 'Development of a molecular genetic classification of breast cancer'.

Fluorescence *in situ* hybridization (FISH) was performed using the *ETV6* FISH DNA Probe Split Signal (Dako, Glostrup, Denmark). This probe is composed of two ready-to-use FISH DNA probes: a Texas Red-labelled DNA probe ('ETV6-Upstream') covering 264 kb telomeric to the *ETV6* breakpoint cluster region, and a fluorescein-labelled DNA probe ('ETV6-Downstream') covering 483 kb centromeric to the *ETV6* breakpoint cluster region. FISH was performed according to the protocol described by Lambros *et al.*[20] After being deparaffinized and rehydrated, the slides were treated with a pepsin solution for 5–5.5 min and then denatured in the presence of the probe on a plate at 85°C for 5 min. Finally, the slides were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) and mounted with Vectashield (Vector Laboratories, Peterborough, UK).[20]

The translocation 'fusion probe' for the t(12;15) *ETV6/NTRK3* translocation was generated as previously described.[20,23] Briefly, a probe telomeric to *ETV6* on 12p comprising the bacterial artificial chromosome (BAC) clones RP11-434C1 and RP11-407p10 was designed and labelled with biotin as reported by Lambros *et al.*[20] A probe centromeric to *NTRK3* composed of BAC clones RP11-114I9 and RP11-730G13 was designed and labelled with digoxigenin as previously described.[20] All BACs were purchased from BACPAC resources, Children's Hospital Oakland (Oakland, CA, USA). Both components of the translocation 'fusion probe' were co-precipitated together with 30 μg of human COT-1 DNA (Invitrogen, Paisley, UK) and 10 μg of salmon sperm DNA (Invitrogen). Slides were de-waxed and treated with the pretreatment buffer (SPOT-light tissue pre-treatment kit; Zymed, South San Francisco, CA, USA) on a hotplate for 17 min at 98°C according to the manufacturer's protocol.[20] Subsequently, the treated sections were digested with pepsin for 5.5 min, washed with water, dehydrated, air-dried and left to hybridize overnight with the translocation 'fusion probes' as previously described.[20] Sections were rinsed in 0.5× NaCl and sodium citrate (SSC) at room temperature and then washed in 0.5× SSC for 7 min at 78°C. The slides were developed with a mixture of 1 μl of Avidin-Cy3/1 μl of anti-digoxigenin-fluorescein diluted in 1:200 in phosphate-buffered saline (PBS)/2% bovine serum albumin for 50 min in the dark at room temperature, and washed with PBS/0.025% Tween 20.[20] Finally, sections were mounted with anti-fade medium containing DAPI.

FISH signals were analysed with a Zeiss Axioplan2 microscope, equipped with an Applied Imaging charge coupled device (CCD) camera (Applied Imaging Diagnostic Instruments, HR070-CMI, Model 4912-5010; Newcastle upon Tyne, UK) and Cytovision software, version 2.81 (Applied Imaging International, Newcastle upon Tyne, UK). Multiple focal planes were stacked for the analysis. In addition, all multiple focal planes were collected sequentially in three channels (DAPI, fluorescein isothiocyanate, Texas Red) on a TCS SP2 confocal microscope (Leica, Milton Keynes, UK) or Zeiss Axioplan II microscope (Carl Zeiss, Oberkochen, Germany) coupled with a CCD camera (Cohu Inc., San Diego, CA, USA). For each case, at least 60 non-overlapping, interphase nuclei were analysed. Cases were considered as harbouring an *ETV6* gene rearrangement if >10% nuclei displayed 'split apart signals' (i.e. red and green signals were separated by a distance greater than the size of two hybridization signals). All cases harbouring *ETV6* split apart signals were subsequently analysed with the 'fusion probes' *ETV6/NTRK3*.

Results

The three SCs showed the typical morphology, all being composed of an admixture of solid, microcystic and glandular areas. As expected,[14,18–21] the secretory carcinomas analysed in this study harboured *ETV6* 'split apart' signals in the vast majority of the cells (Figure 1). With the translocation 'fusion probes', the presence of t(12;15) translocations in the three SCs was confirmed (Figure 1).

Four ACCs showed the typical morphology, as described by Damiani *et al.*,[4] and the remaining two cases (cases 5 and 6) were composed of cells with more abundant, granular eosinophilic-to-amphophilic cytoplasm arranged in sheets; nuclei were vesicular, more pleomorphic, with conspicuous nucleoli. These cases were classified as high-grade/poorly differentiated ACCs.[4] None of the ACCs showed any definite evidence of *ETV6* gene rearrangement (Figure 2). Although one case of ACC showed suboptimal results (case 6), none of the cells displayed any 'split apart' signals. Interestingly, additional copies of chromosome 12p were

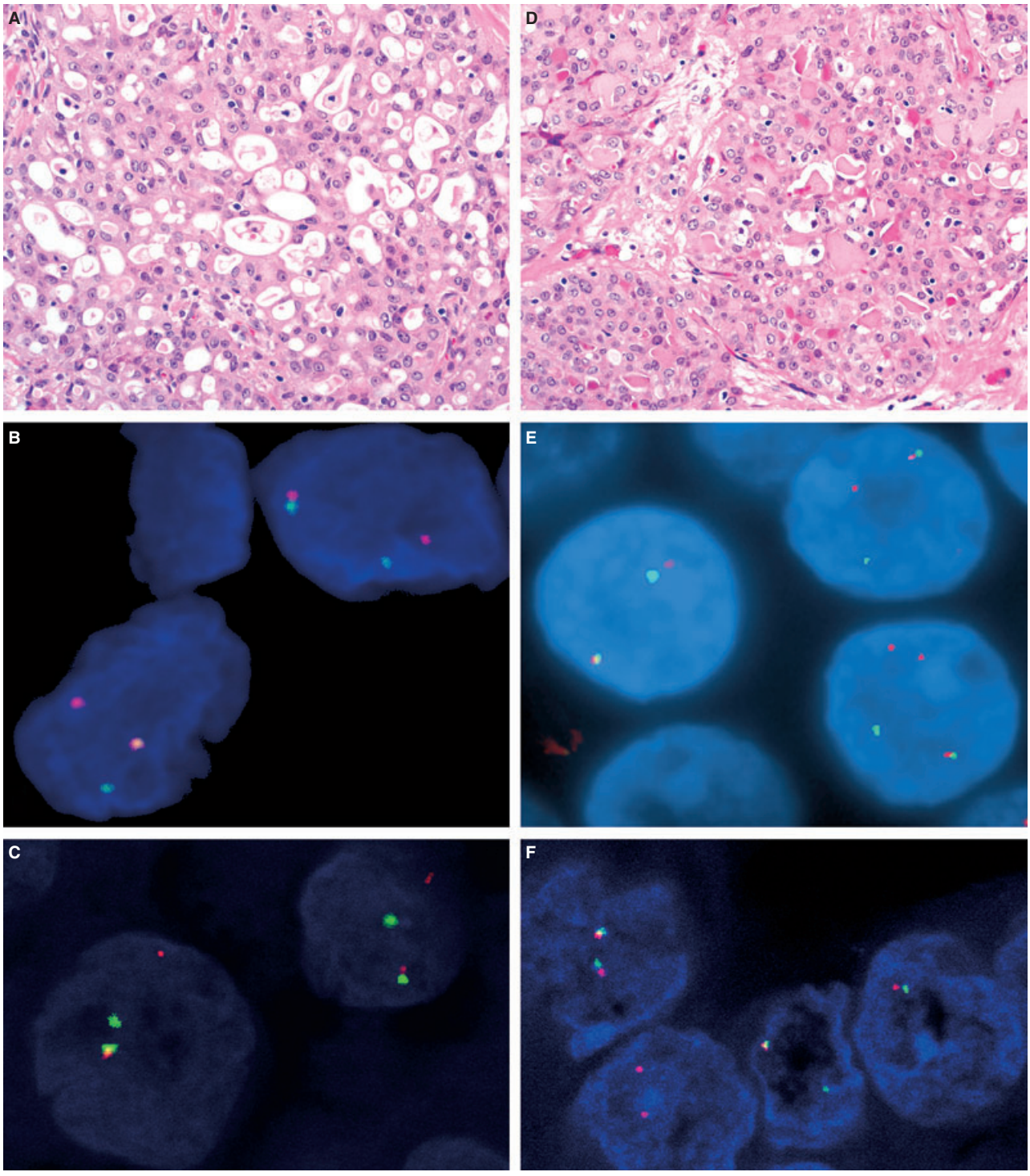

Figure 1. Secretory carcinomas harbour rearrangements of the *ETV6* gene. A typical secretory carcinoma (**A**) characterized by microcystic areas composed of cuboidal and polygonal cells, with bubbly, clear and eosinophilic cytoplasm. Fluorescence *in situ* hybridization (FISH) with *ETV6* split-apart probes demonstrating the presence of *ETV6* gene rearrangement (**B**) and with *ETV6/NTRK3* 'fusion probes' demonstrating the t(12;15) fusion (**C**). Another example of a typical secretory carcinoma (**D**) composed of microcystic areas harbouring *ETV6* split-apart signals (**E**) and the fusion t(12;15) (**F**). In **C** and **F**, *NTRK3* signals are seen in green (fluorescein) and *ETV6* signals are seen in red (Cy3); note the presence of fusion signals in the nuclei of neoplastic cells. (**A, C,** H&E; **B, C, F,** FISH images captured with a TCS SP2 confocal microscope; **E,** FISH image captured with a Zeiss Axioplan II microscope.)

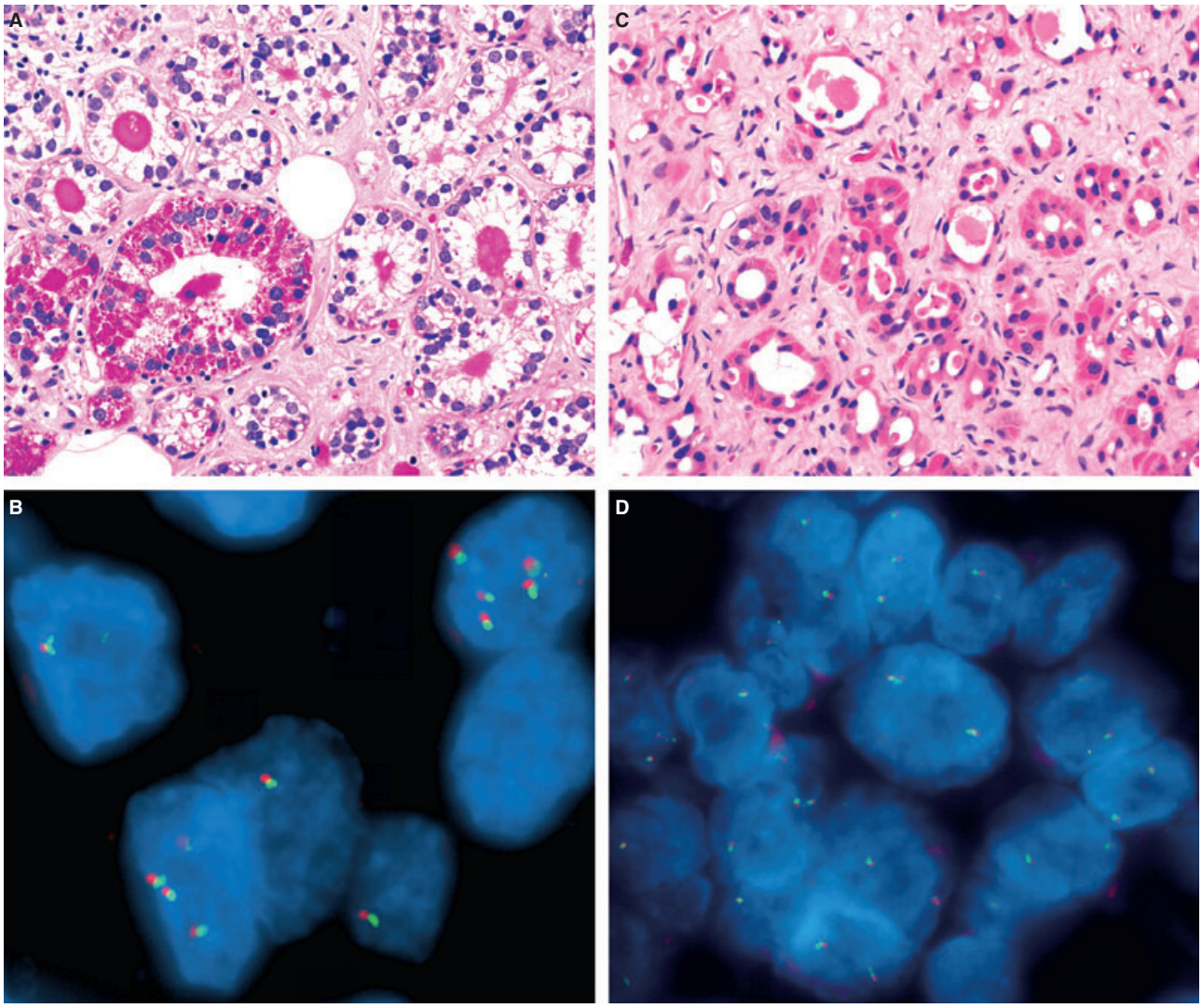

Figure 2. Acinic cell carcinomas of the breast do not harbour *ETV6* gene rearrangements. A typical acinic cell carcinoma (**A**) lacking rearrangements of the *ETV6* gene but harbouring an increase in 12p copy numbers (**B**). An acinic cell carcinoma predominately composed of oxyphilic cells (**C**) with no split-apart signals (**D**). (**A, C,** H&E; **B,** fluorescence *in situ* hybridization (FISH) image captured with a Zeiss Axioplan II microscope; **D,** FISH image captured with a Zeiss Axioplan II microscope.)

identified in the non-modal population of two ACCs, but no *ETV6* 'split apart' signals were found.

Discussion

In this study, we have demonstrated that both typical and high-grade ACCs of the breast lack the *ETV6* 'split apart' signals and are therefore unlikely to harbour the t(12;15) *ETV6/NTRK3* translocation. On the other hand, the three samples of SCs analysed harboured this translocation. Our results rule out the presence of *ETV6* gene rearrangements in ACCs and, therefore, the presence of the t(12;15) *ETV6/NTRK3* translocation found in SCs of the breast. The *ETV6* 'split apart' probe cannot exclude other rearrangements of the *NTRK3* gene; however, no partner other than *ETV6* has been reported in balanced chromosomal translocations involving *NTRK3* in breast cancer.

The *ETV6/NTRK3* translocation is not entirely specific to SCs.[24] In fact, this translocation is reported to be found in congenital fibrosarcomas, cellular mesoblastic nephromas and acute myeloid leukaemia.[24,25] Interestingly, in the context of breast malignancies, this translocation appears to be restricted to SCs.[23,26] Two studies have investigated the presence of t(12;15)(p13;q25) in breast carcinomas other than SCs

by means of FISH.[23,26] In one study, this translocation was not found in any of the 201 non-secretory breast carcinomas,[23] whereas in a series of 354 cases, Letessier *et al.*[26] found five *ETV6* gene rearrangements in non-secretory breast carcinomas; however none of them showed expression of the *ETV6/NTRK3* fusion product, suggesting that the *ETV6* rearrangement was not the result of the t(12;15)(p13;q25) translocation. In this study, all cases with 'split apart' *ETV6* signals were typical SCs and the presence of the t(12;15) *ETV6/NTRK3* translocation was confirmed in all cases.

As emphasized by Hirokawa *et al.*,[22] ACCs and SCs do have some similarities, including the low-grade malignant nature of the neoplasms, the presence of a complex admixture of solid, microcystic and tubular growth patterns, the granular cytoplasm of neoplastic cells, the preferential hormone receptor-negative status and the positivity for amylase, lysozyme and chymotrypsin.

On the other hand, SC and ACC differ in terms of their clinical presentation: no cases of ACC affecting prepubertal patients or males have been reported to date.[1–10] Furthermore, cytologically, SCs are remarkably different from ACC, given that the cells of the former are characterized by abundant production of milk-like, secretory material, often with intracytoplasmic vacuolization, whereas the cells of ACC are finely granular, sometimes resembling Paneth cells. In addition, the positivity for cytokeratin 17 in SCs (J S Reis-Filho, unpublished data) and the absence of *ETV6* gene rearrangements in ACCs provide strong circumstantial evidence to suggest that ACC of the breast is not a variant of SC of the breast and should be considered a separate entity.

An alternative explanation for our findings is that ACC and SC form a biological continuum and that ACC can 'progress' to SC with the acquisition of the t(12;15)(p13;q25) *ETV6/NTRK3* translocation. However, the fact that pure ACC has not been reported in prepubertal and male patients, that typical areas of ACC have not been reported in association with SCs affecting prepubertal and male patients and the absolute lack of *ETV6* gene rearrangements in the six cases of ACC analysed here suggest that this alternative hypothesis is unlikely.

In conclusion, our results strongly support the concept that SCs and ACCs are distinct entities and should be recorded separately in breast cancer taxonomy schemes. Despite the rarity of these lesions and their reported low-grade malignant potential, further studies analysing in detail the molecular genetic features and expression profiles of a large series of these lesions are warranted.

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