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Genomic and immunohistochemical analysis of adenosquamous carcinoma of the breast

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Breast adenosquamous carcinomas are rare tumours characterized by well-developed gland formation intimately admixed with solid nests of squamous cells immersed in a highly cellular spindle cell stroma. A low-grade variant has been described that is associated with a better prognosis. Here we studied five cases of adenosquamous carcinomas to determine their genetic profiles and to investigate whether the spindle cell component of these cancers could at least in part stem from the glandular/epithelial components. Five adenosquamous carcinomas of the breast were subjected to (1) immunohistochemical analysis, (2) microdissection and genetic analysis with a high-resolution microarray comparative genomic hybridization platform, and (3) chromogenic *in situ* hybridization. All cases displayed a triple-negative immunophenotype, consistently expressed 'basal' keratins and showed variable levels of epidermal growth factor receptor expression. Microarray comparative genomic hybridization analysis of two of the cases revealed multiple low-level gains and losses affecting several chromosomal arms. Case 1 displayed gains of the whole of chromosome 7, and case 2 harboured a focal, high-level amplification of 7p12, encompassing the epidermal growth factor receptor gene, which was associated with strong and intense membranous epidermal growth factor receptor expression. Chromogenic *in situ* hybridization revealed that the genetic features found in the epithelial cells were also present in a minority of the spindle cells of the stromal component, in particular in those near the epithelial clusters, indicating that some of the spindle cells are clonal and derived from the epithelial component of the tumour. In conclusion, breast adenosquamous carcinomas are triple-negative cancers that express 'basal' keratins. These tumours harbour complex genetic profiles. Some of the spindle cells in adenosquamous carcinomas are derived from the epithelial component, suggesting that adenosquamous carcinomas may also be part of the group of metaplastic breast carcinomas with spindle cell metaplastic elements.
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Adenosquamous carcinomas of the breast are rare tumours, included in the last edition of the World Health Organization (WHO) classification of breast cancers[1,2] as a subtype of metaplastic carcinoma. They are characterized by well-developed gland/tubule formation intimately admixed with solid nests of squamous cells in a spindle cell background. A low-grade variant was described in 1987 by Rosen and Ernsberger,[3] who highlighted that, despite the presence of metaplastic elements, these tumours displayed a low-grade pattern, bearing some resemblance to tubular carcinomas. In agreement with their low-grade morphological features, the majority of low-grade adenosquamous carcinomas have been described to have an excellent prognosis, with a low incidence of lymph node metastasis.[1,4] A proportion of cases, however, can behave in a locally aggressive manner.

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At gross examination, low-grade adenosquamous carcinomas tend to display a stellate or infiltrative configuration, with poorly defined borders. Microscopically, the carcinomatous component is characterized by small glandular structures, with rounded rather than angulated contours, and solid cords of epithelial cells, which may contain squamous cells, squamous pearls or squamous cyst formation. The invasive neoplastic component typically shows long, slender extensions at the periphery and infiltrate in between normal breast structures, features which have been associated with inadequate local excision and high incidence of recurrence.[1] Clusters of lymphocytes are often observed at the periphery. Furthermore, the association between these tumours and adenomyoepithelioma and sclerosing proliferative lesions has been reported.[4,5]

The epithelial structures of adenosquamous carcinomas are often immersed in a highly cellular spindle cell stroma. Stromal cells appear to merge with the epithelial cells and, in fact, in some cases, spindle cell metaplasia is recognized and some have suggested that the stroma should be considered as part of the neoplastic component.[1,3–5] As emphasized in the first description of the low-grade variant, however, in some instances, it may be 'difficult to distinguish between spindle cell metaplasia and ordinary stroma'.[3] Furthermore, so far, the available evidence in support of the concept that some of the stromal spindle cells are indeed metaplastic, that is, derived from the epithelial cells, remains restricted to phenotypic analyses (ie, histopathological and immunohistochemical). Ultra-structural features of the adenocarcinomatous and squamous cells have been described in detail.[4,6] However, probably due to characteristics of the specimen subjected to electron microscopy, the stroma was reported to consist 'primarily of numerous collagen fibrils'. Few immunohistochemical studies have described negativity for hormone receptors[5–7] and a high prevalence of HER2 membrane staining (46%) in tumours cells;[7] no genome-wide molecular genetic analysis of low-grade adenosquamous carcinomas has been performed to our knowledge.

Here we report on five cases of adenosquamous carcinoma of the breast, which were subjected to high-resolution microarray comparative genomic hybridization, followed by chromogenic *in situ* hybridization and immunohistochemistry. The aims of this study were twofold: (1) to characterize the genetic profiles of adenosquamous carcinomas and (2) to determine if cells of the spindle cell component harbour the genetic aberrations found in the glandular/epithelial components.

Materials and methods

Cases

Five cases diagnosed as adenosquamous carcinoma of the breast were retrieved from the files of the Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA. Samples were anonymized before analysis. Ethical approval was available.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were cut at 3 μm and mounted on silane-coated slides. Immunohistochemistry was performed as previously described[8] using antibodies raised against estrogen receptor (ER), progesterone receptor (PR), HER2 and 'basal-like' markers (epidermal growth factor receptor (EGFR), cytokeratin (CK) 5/6, CK14, CK17). The antibody sources, dilutions, pre-treatment protocols and detection methods are listed in Table 1. Positive and negative controls (omission of the primary antibody and IgG-matched serum) were included for each immunohistochemical run. All markers were scored separately in the epithelial/glandular and spindle cell components of the tumours. For ER and PR, only nuclear reactivity was considered specific and the percentage of positive cells was estimated for each marker. For CKs, any cytoplasmic staining in morphologically unequivocal neoplastic cells was recorded as positive. HER2 was evaluated according to current ASCO/CAP guidelines.[9] EGFR was scored as previously described.[10]

Table 1 Summary of antibody sources, dilutions, pretreatment protocols and detection methods used

Primary antibody	*Supplier*	*Clone/code*	*Antigen retrieval*	*Dilution*	*Detection*
ER	Dako	ID5/M7047	2 min PC	1:40	ABC
PR	Dako	PgR636/M3569	2 min PC	1:200	ABC
HER2	Dako	K5207	40 min PTM	Neat	Envision
EGFR	Invitrogen	31G7/SKU#28-0005	10 min 0.1% pronase	1:50	ABC
CK5/6	Chemicon	D5/16 B4/MAB1620	18 min MW	1:600	ABC
CK14	Vector Labs	LL002/VP-C410	18 min MW	1:40	ABC
CK17	Dako	E317/M7046	18 min MW	1:100	ABC
SMA	Dako	1A4/0851	None	1:300	ABC-AP

ABC: avidin-biotin complex; ABC-AP: avidin-biotin complex and alkaline phosphatase; CK: cytokeratin; CT: chymotrypsin; EGFR: epidermal growth factor receptor; ER: estrogen receptor; MW: microwave oven; PC: pressure cooker; PR: progesterone receptor; PTM: Thermo Fisher pre-treatment module; SMA: smooth muscle actin.

Microdissection and DNA Extraction

All tumours were microdissected to ensure >90% of purity of neoplastic cells. Microdissection was performed with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan) from 10 consecutive 8-μm-thick sections stained with nuclear fast red as previously described.[11] DNA was extracted using the DNeasy Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. DNA concentration was measured with the PicoGreen® assay as per the manufacturer's instructions (Invitrogen, Paisley, UK).[11]

Microarray Comparative Genomic Hybridization

The microarray comparative genomic hybridization platform used for this study was constructed at the Breakthrough Breast Cancer Research Centre and comprises ~32 000 bacterial artificial chromosome (BAC) clones tiled across the genome. This type of BAC array platform has been shown to be as robust as and to have comparable resolution with high-density oligonucleotide arrays.[12–14] Labelling, hybridization, washes, image acquisition and data normalization were carried out as previously described.[11] The final data set comprised 31 367 clones with unambiguous mapping information according to the hg19 of the human genome (http://www.ensembl.org). Copy number changes were categorized as gains, losses or amplifications according to previously validated thresholds for each clone.[15] Threshold values were chosen to correspond to three standard deviations of the normal ratios obtained from the filtered clones mapping to chromosomes 1–22, assessed in multiple hybridizations between DNA extracted from a pool of male and female blood donors as previously described[16,17] (Log2 ratio of ±0.12). Low-level gain was defined as a smoothed Log2 ratio of between 0.12 and 0.45, corresponding to approximately 3–5 copies of the locus, whereas gene amplification was defined as having a Log2 ratio >0.45, corresponding to more than five copies.

Chromogenic *In Situ* Hybridization

Chromogenic *in situ* hybridization was used to validate the results of microarray comparative genomic hybridization analysis and to investigate whether the detected genetic aberrations were present in the distinct components of the tumours. Ready-to-use digoxigenin-labelled SpotLight amplification probe for *EGFR* (Zymed, CA, USA) and biotin-labelled centromeric probe for chromosome 7 (CEP7; Zymed) were used. Formalin-fixed, paraffin-embedded sections were cut at 2 μm and mounted on silane-coated slides. Tissue pre-treatment, hybridizations and washes were performed as previously described.[18] Signals were counted in the nuclei of 60 morphologically unequivocal tumour cells (epithelial and stromal components) and normal cells for EGFR probe and CEP7. Amplification was defined as the presence of large gene clusters or >5 gene copies in >50% of the neoplastic cells.

Simultaneous Immunohistochemistry and Chromogenic *In Situ* Hybridization

Formalin-fixed, paraffin-embedded sections were cut at 2 μm and mounted on silane-coated slides. Immunohistochemistry was performed with an antibody against smooth muscle actin (SMA). Details of the antibody source and dilution, and detection method are listed in Table 1. Antibody binding was detected using an alkaline phosphatase-conjugated avidin–biotin complex (ABC) kit (Vector Labs, CA, USA) and Vector Red chromogen (Vector Labs). Slides were incubated for 30 min in secondary antibody and ABC, and for 20 min in chromogen. Immediately after the immunohistochemical reaction, sections were subjected to chromogenic *in situ* hybridization analysis following the same protocol as described above. Signals were scored as described above.

Results

Cases

All patients were female and predominantly postmenopausal, the age at diagnosis ranging from 54 to 76 years (mean = 70; median = 72). All but one tumour were staged as pT2, with maximum diameter ranging from 1.5 to 3.7 cm (mean = 2.7 cm; median = 2.6 cm). Axillary nodal status was available only for one case (case 2), and no metastatic deposits were observed.

Histopathological Analysis

Histopathological review was performed by three of the authors (FG, SB, JR-F). All cases were composed of varying proportions of adenocarcinomatous, squamous and stromal components and four cases (cases 1, 3, 4 and 5) entirely fulfilled the criteria proposed by Rosen and Ernsberger[3] for low-grade adenosquamous carcinomas (Figure 1 and Supplementary Figures 1, 2, 3 and 4). These four tumours were characterized by infiltrative borders (Figure 1a) and neoplastic glands tended to infiltrate between ducts and into lobules (Figure 1b); the epithelial component was in the form of small tubules, cell clusters and cords (Figure 1c) with focal squamous differentiation (Figure 1d), haphazardly arranged in an infiltrative spindle cell background. The glandular/epithelial cells tended to subtly merge with the spindle cells (Figure 1e). Lymphocytic aggregates were present in all tumours and conspicuous at the periphery of three cases (Figure 1e). Case 5 was

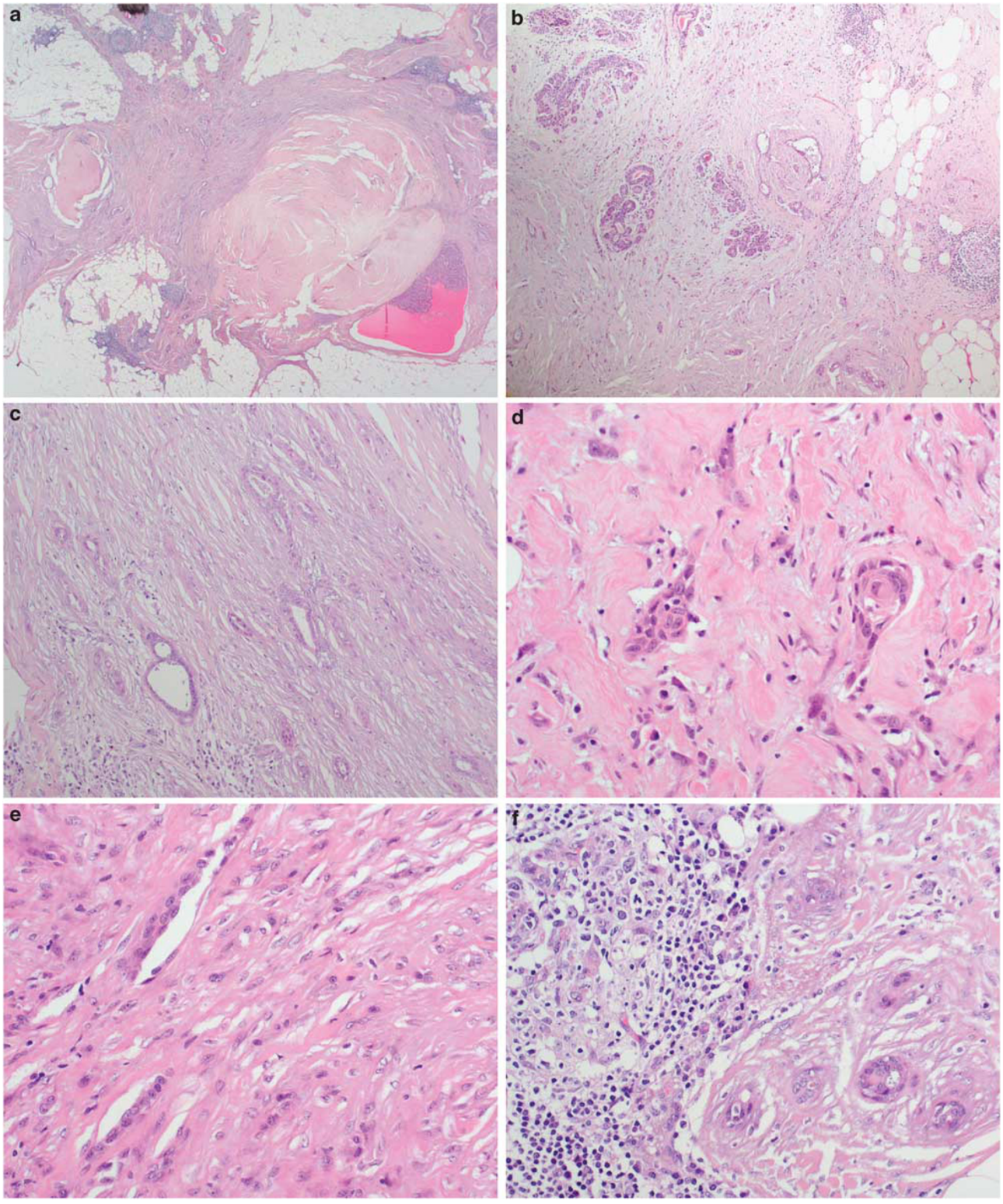

Figure 1 Histological features of cases 1, 3, 4 and 5. Tumour borders were infiltrative (**a**, case 5) and neoplastic cells infiltrated the normal breast structures (**b**, case 1). Small tubules and cell clusters (**c**, case 5) with focal squamous differentiation (**d**, case 4) were arranged in haphazard manner in a spindle cell stroma. The spindle cells often appeared to merge with the epithelial cells (**e**, case 1). Lymphocytic infiltrate was conspicuous in three cases (**f**, case 3). Additional representative micrographs of cases 1, 3, 4 and 5 are available in Supplementary Figures 1, 2, 3 and 4, respectively.

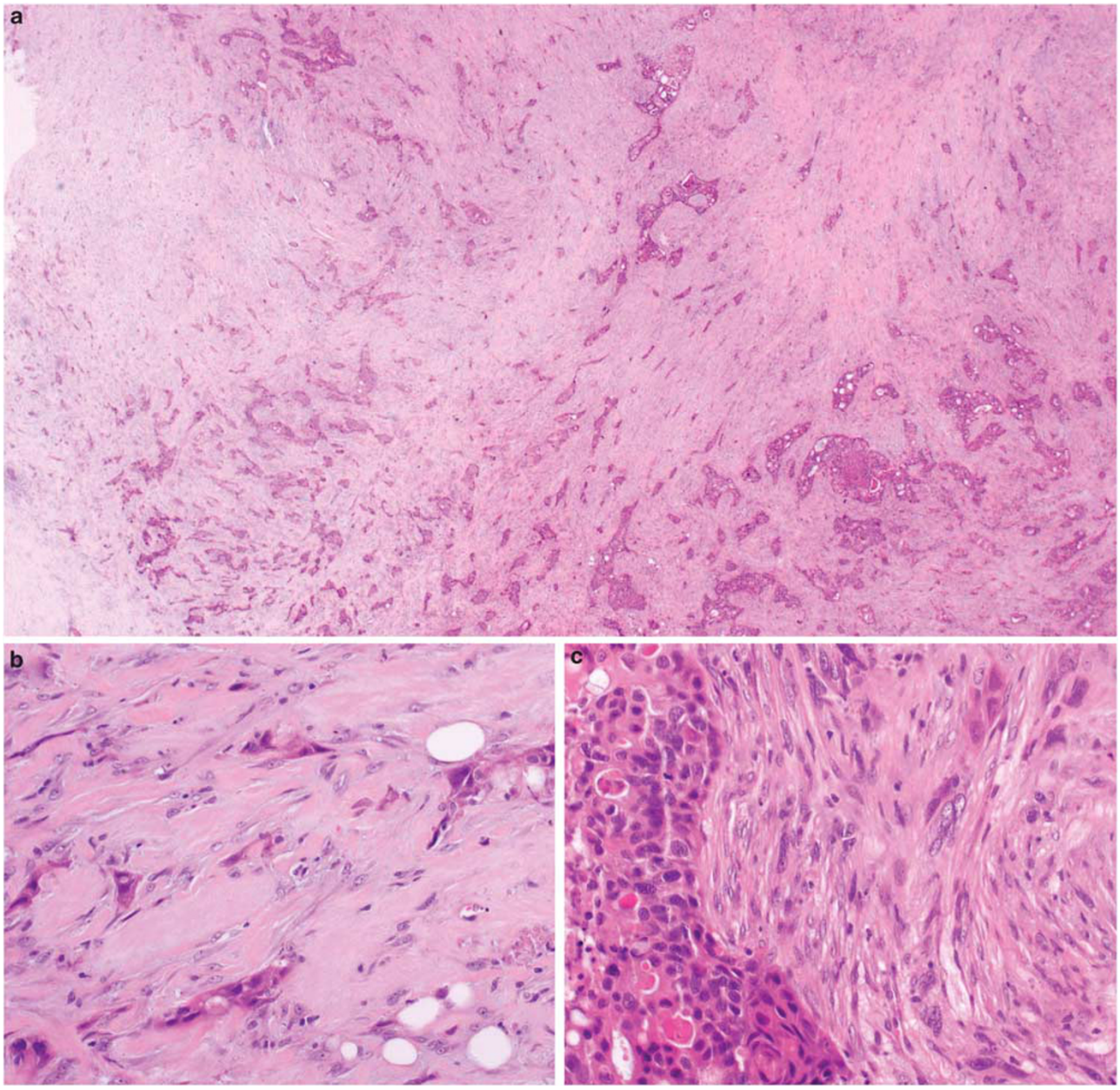

Figure 2 Histological features of case 2. A transition from areas typical of low-grade adenosquamous carcinomas (**a**, at left side, and **b**) to high-grade areas, which also comprised overtly malignant spindle cells (**a**, at right side, and **c**), was observed. Additional representative micrographs of case 2 are available in Supplementary Figure 5.

associated with a sclerosing papillary lesion (Figure 1a), which may have possibly been the substrate from which the invasive tumour originated.[5] The remaining case (case 2) had areas that displayed the hallmark features of a low-grade adenosquamous carcinoma (Figure 2a and b and Supplementary Figure 5); however, a gradual transition to high-grade features with an overtly malignant spindle cell component was observed (Figure 2a and c and Supplementary Figure 5). This case is consistent with previous description of progression from low-grade adenosquamous carcinomas to a high-grade metaplastic carcinoma.[5,19]

Immunohistochemistry

Results of the immunohistochemical analysis are summarized in Table 2 and shown in Figure 3. Immunohistochemical profiles were very similar across the different cases. All tumours were of triple-negative phenotype (ie, ER-, PR- and HER2-negative)

Table 2 Summary of immunohistochemical analysis

Marker	*Case 1*		*Case 2*		*Case 3*		*Case 4*		*Case 5*	
Component	*Epithelial*	*Stromal*	*Epithelial*	*Stromal*	*Epithelial*	*Stromal*	*Epithelial*	*Stromal*	*Epithelial*	*Stromal*
ER	–	–	–	–	–	–	–	–	–	–
PR	–	–	–	–	–	–	–	–	–	–
HER2	–	–	–	–	–	–	–	–	–	–
CK5/6	+++	±	+++	±	+++	±	+++	±	+++	±
CK14	+++	±	+++	±	+++	±	+++	±	+++	±
CK17	+++	±	+++	±	+++	±	+++	±	+++	±
EGFR	1+	–	3+	3+	2+	2+	–	–	3+	1+

+++: diffusely positive; +: positive; ±: focally positive; –: negative; CK: cytokeratin; EGFR: epidermal growth factor receptor; ER: estrogen receptor; PR: progesterone receptor.

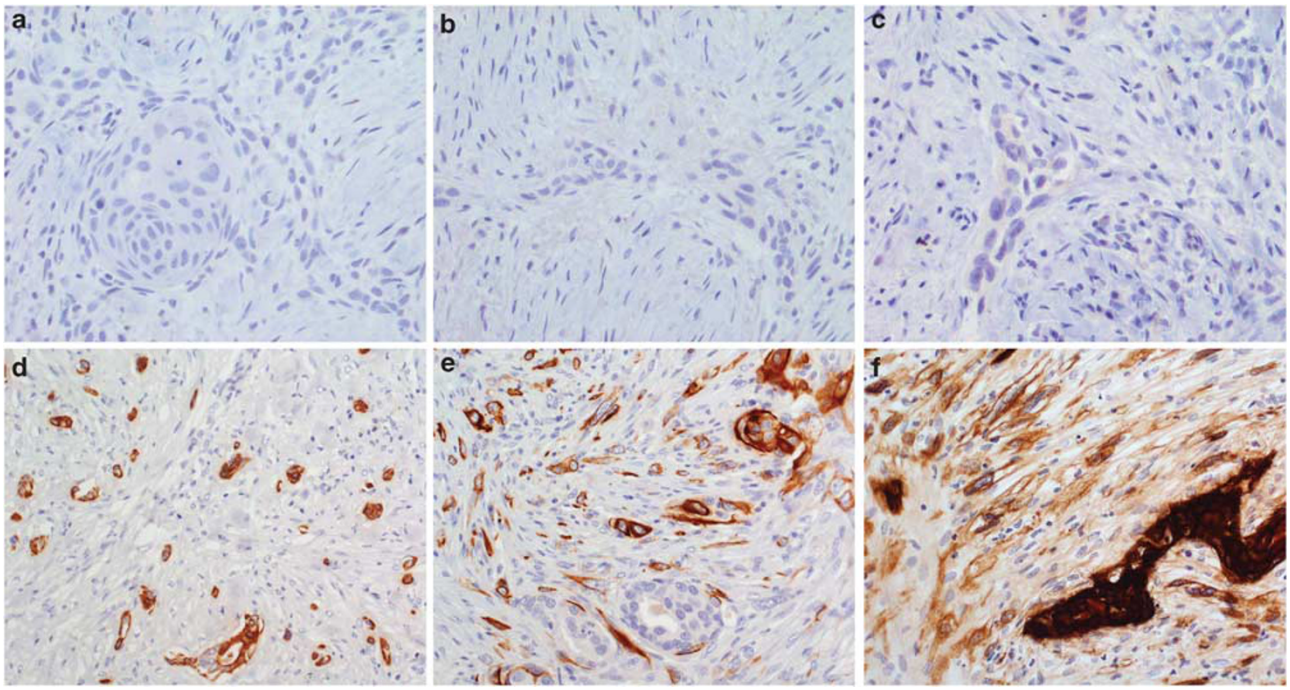

Figure 3 Immunohistochemical features. All tumours were negative for estrogen (**a**) and progesterone (**b**) receptors, and HER2 (**c**). Consistent positivity for basal cytokeratins (CKs), including CK17 (**d**) and CK14 (**e**), was observed. EGFR overexpression was present in case 2, with strong staining in spindle cells of the high-grade component (**f**).

(Figure 3a, b and c) with consistent expression of basal markers. Positive reactivity for CK5/6, CK14 (Figure 3d) and CK17 (Figure 3e) was present in all cases, with a diffuse strong staining in the epithelial cells, whereas only scattered stromal cells were positive (Supplementary Figures 1f, 2f, 3f, 4f and 5k and l). EGFR was overexpressed in cases 2, 3 and 5, with predominant staining in the epithelial cells. In case 2, the high-grade component displayed a strong staining for EGFR, including in the stromal spindle cells (Figure 3f). The immunohistochemical profile of all cases was consistent with that of basal-like breast cancers, according to the microarray gene expression profiling-validated immunohistochemical definition proposed by Nielsen *et al*[20] (ie, lack of ER and HER2, and expression of CK5/6 and/or EGFR), which has a specificity of 100% for basal-like breast cancers.

Microarray Comparative Genomic Hybridization

Optimal quality DNA for microarray comparative genomic hybridization experiments was obtained only from cases 1 and 2. In agreement with its low-grade morphological features,[21] case 1 displayed a rather simple molecular karyotype (Figure 4a and c), comprising gains of 6p, 7pq and 8q, losses of 1p, 6p, 6q, 8p and 9p, and no amplifications. Case 2 displayed a greater degree of genetic complexity

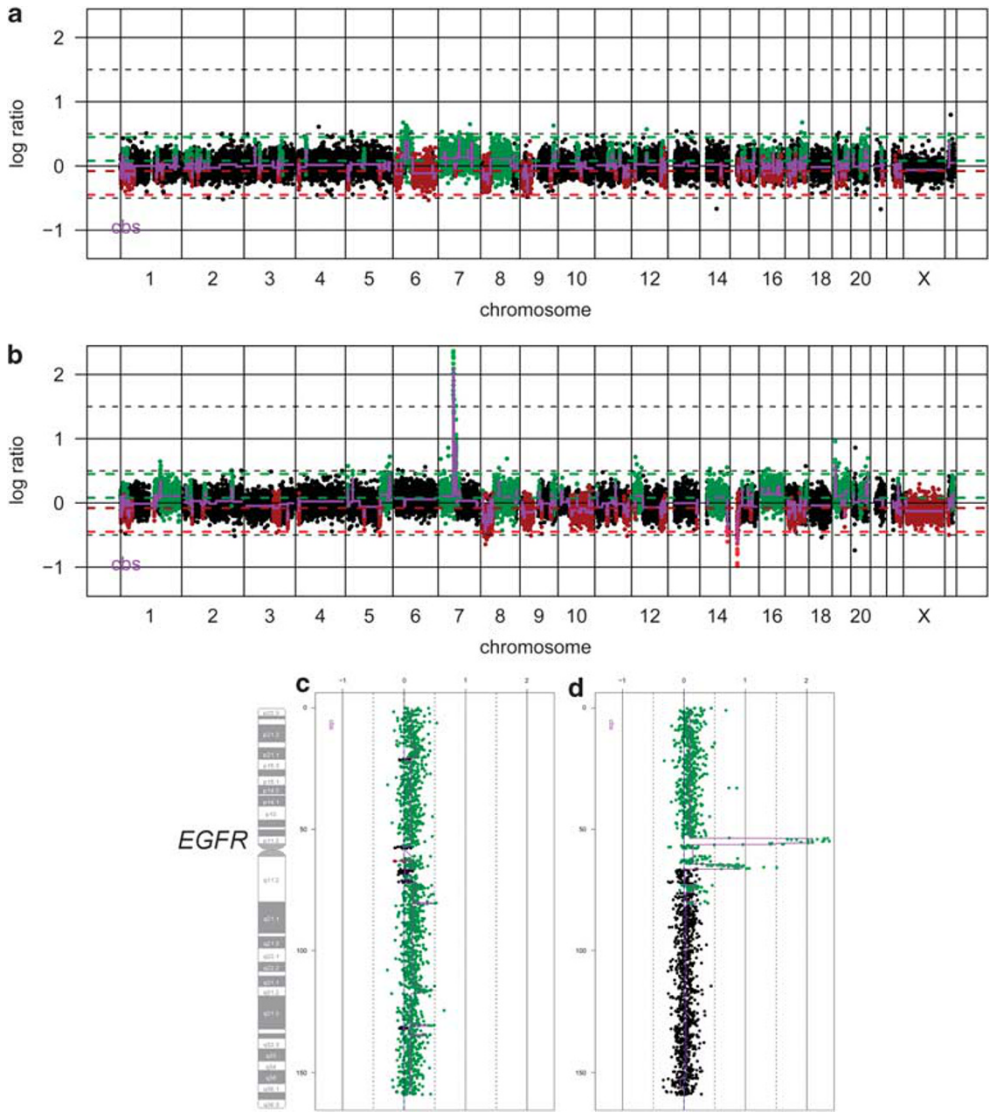

Figure 4 Genome plots and chromosome 7 plots of case 1 (**a** and **c**) and case 2 (**b** and **d**). Case 1 displayed a rather simple pattern of genomic aberrations, whereas case 2 showed a greater complexity, with a complex firestorm pattern,[22] with two regions of high-level amplification in chromosome 7, one of which encompassed the *EGFR* gene locus. In the genome plots (**a** and **b**), CBS (circular binary segmentation) $\log_2$ ratios are plotted on the *y* axis against each clone according to genomic location on the *x* axis. BACs categorized as displaying genomic gains or amplification are plotted in green and those categorized as genomic losses in red. CBS $\log_2$ ratios are plotted in purple. In the chromosome 7 plots (**c** and **d**), CBS $\log_2$ ratios are plotted on the *x* axis against each clone according to genomic location on the *y* axis. BACs categorized as displaying genomic gains or amplification are plotted in green and those categorized as genomic losses in red.

(Figure 4b and b). Its genomic profile was characterized by a complex 'firestorm' pattern,[22] with two amplification peaks on chromosome 7. Gains of 1q, 5q, 7p, 8q, 12p, 14q, 16p, 16q and 18pq, losses of 1p, 3q, 8p, 9p, 12q, 17p, 17q, 22q and Xpq, and high-level amplification of 7p11.2, encompassing the *EGFR* gene, and 7q11.21 were observed.

Chromogenic *In Situ* Hybridization

To validate microarray comparative genomic hybridization findings and study the presence of the detected copy number changes in the distinct components of the tumours, we performed chromogenic *in situ* hybridization for selected regions harbouring gains or amplification. Microarray comparative genomic hybridization analysis revealed that in case 1 the whole of chromosome 7 harboured gains (Figure 4c). Chromogenic *in situ* hybridization for the *EGFR* gene, which maps to 7p11.2, and CEP7 confirmed this finding, showing averages *EGFR* and CEP7 copy numbers consistent with gain of a copy of chromosome 7 in the epithelial cell clusters and also in the surrounding stromal tumour cells (Table 3). Microarray comparative genomic hybridization analysis of case 2 revealed gains of 7p-q11.21, encompassing the centromere and a high-level

Table 3 Summary of copy number status for *EGFR* gene and CEP7 by chromogenic *in situ* hybridization of cases 1 and 2

Case	*Probe*	*Normal cells*	*Epithelial tumour cells*	*Stromal tumour cells*
1	*EGFR*	1.52	2.34	2.38
	CEP7	1.46	2.31	2.40
2	*EGFR*	1.55	Large gene clusters	Large gene clusters
	CEP7	1.51	2.46	2.3

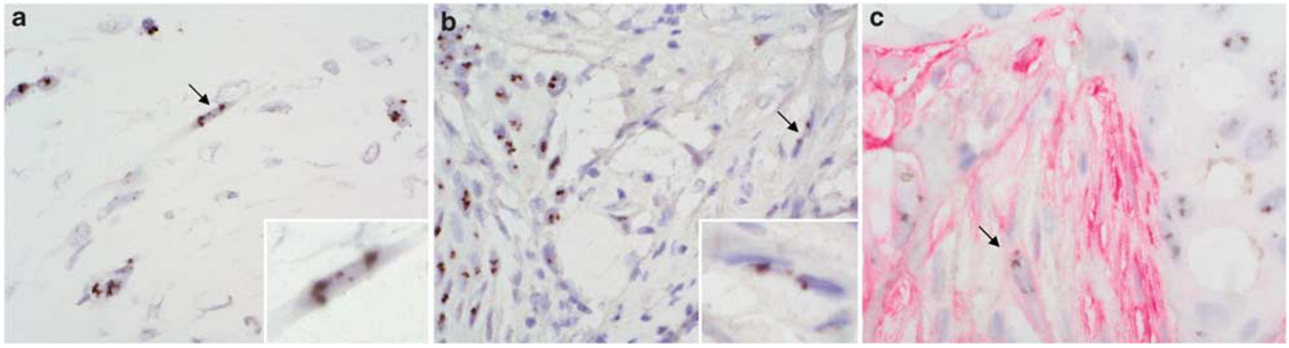

Figure 5 Chromogenic *in situ* hybridization (**a** and **b**) and simultaneous immunohistochemistry and chromogenic *in situ* hybridization (**c**) of case 2. Chromogenic *in situ* hybridization was performed with a probe for the *EGFR* gene, and low-grade (**a**) and high-grade (**b**) areas are depicted. In **c**, simultaneous immunohistochemistry and chromogenic *in situ* hybridization were performed with antibodies against smooth muscle actin and a probe for *EGFR*, respectively. Note that clusters of epithelial cells and some of the spindle cells (arrows and insets in **a** and **b**) of the stromal component display large *EGFR* gene clusters. Note that other stromal cells display only two copies of *EGFR*. In addition, spindle-shaped cells (arrow, **c**) display large *EGFR* gene clusters (brown) and express actin (red).

amplification of *EGFR* (Figure 4d). In this case, chromogenic *in situ* hybridization showed that not only the epithelial cells, but also a minority of the spindle cells in the stromal component, in particular those near the epithelial clusters, harboured *EGFR* amplification (Figure 5a and b, Table 3). It should be noted that spindle cells harbouring *EGFR* amplification were found both in the low- and high-grade areas of this tumour (Figure 5a and Supplementary Figures 5q to v). In addition, CEP7 chromogenic *in situ* hybridization showed gains of the centromeric region in the epithelial and surrounding stromal cells (Table 3).

Simultaneous Immunohistochemistry and Chromogenic *In Situ* Hybridization

To further show that some of the stromal spindle cells in case 2 harboured the same genetic aberrations identified in the glandular/epithelial components, we performed simultaneous immunohistochemistry with an antibody against SMA and chromogenic *in situ* hybridization with *EGFR* probe. As expected, the great majority of epithelial/glandular cells displayed large gene clusters and did not express SMA (Figure 5c, right side). However, a minority of the spindle cells displayed large gene clusters and expressed SMA (Figure 5c, left side), indicating that neoplastic epithelial cells underwent some degree of epithelial-to-mesenchymal transition (EMT) and acquired a spindle cell phenotype.

Discussion

Adenosquamous carcinoma of the breast is an uncommon histological type of breast cancer, characterized by the presence of well-defined adenocarcinomatous and squamous components. It is accepted that spindle cell metaplasia may occur and a neoplastic spindle cell component coexist with the epithelial components. A low-grade variant has been recognized, which displays a distinct morphology and a better clinical outcome.[1,7] These tumours are still poorly characterized at the molecular level. Here we have described an immunohistochemical and genetic analyses of five cases of adenosquamous carcinoma, four of which were *bona fide* examples of the low-grade variant.

All tumours studied here were of triple-negative phenotype. Negativity for hormone receptors has been previously reported;[5,7] however, in contrast with the results of Drudis *et al*,[7] who reported that 46% of these tumours displayed HER2 overexpression, the five adenosquamous carcinomas studied here consistently lacked HER2 expression and the two cases subjected to microarray comparative genomic hybridization analysis lacked *HER2* gene amplification. These discrepant results are probably due to different scoring methods, given that at that moment[7] HER2 status evaluation was not performed according to the current clinical guidelines.[9] Expression of basal markers was found in all tumours. The immunoprofile of adenosquamous carcinomas

falls into the basal-like molecular subtype according to an immunohistochemical surrogate,[20] which has a specificity of 100% for the identification of basal-like breast cancers defined by microarray gene expression profiling. This is in agreement with the observation that focal squamous metaplasia in invasive ductal carcinomas is strongly associated and predictive of the basal-like phenotype[23,24] and with the fact that >90% of metaplastic breast cancers are of basal-like phenotype by immunohistochemical[25,26] and microarray-based analysis.[27,28] Moreover, our results highlight the heterogeneity of basal-like[2,29,30] and triple-negative[31,32] cancers: although as a group triple-negative and basal-like cancers are associated with a more aggressive clinical behaviour,[33–35] they also encompass rare entities associated with a good prognosis.[27,29–31,36,37]

By performing microarray comparative genomic hybridization followed by chromogenic *in situ* hybridization in two of the cases, we provide direct molecular evidence that a proportion of the spindle cells of the stromal component of adenosquamous carcinomas harbour the same genetic aberrations present in the glandular/epithelial cells (Figure 5 and Table 3) and are indeed derived from the epithelial cells. To corroborate this finding, we have shown that, in case 2, the spindle-shaped cells harbouring *EGFR* amplification expressed SMA (Figure 5c). Consistent with this observation EGFR protein overexpression was observed in the overtly malignant spindle cells of the high-grade areas of this case (Figure 3f). Furthermore, a minority of the spindle cells surrounding the epithelial clusters expressed CKs (Figure 3e). Taken together, our results provide direct evidence to support the hypothesis that at least part of the so-called stromal component of adenosquamous carcinoma is in fact derived from the epithelial/glandular component and that some form of EMT may take place in adenosquamous carcinomas, in a way akin to the EMT described in other forms of metaplastic breast cancer and mixed malignant Mullerian tumours.[27,38,39]

In conclusion, we have shown that adenosquamous carcinomas of the breast, including the low-grade variant, harbour complex genomic profiles, are of triple-negative phenotype, express basal makers and may harbour *EGFR* gene amplification. Furthermore, we have provided direct molecular evidence that a minority of the spindle cells of the stromal component are derived from the epithelial cells, suggesting that some form of EMT may take place in this subtype of metaplastic carcinoma. It should be noted that it is not uncommon for adenosquamous carcinomas to have genuine sarcomatoid stroma, as previously described[5,19] and also observed in the cases described here (Figure 2c). Furthermore, the presence of CK-positive spindle cells in cases of low-grade adenosquamous carcinomas is not uncommon.[5] Taken together, these lines of evidence suggest that low-grade adenosquamous carcinomas may be best classified as tumours displaying squamous and spindle cell metaplasia rather than 'pure epithelial squamous cell carcinomas', as described in the current WHO classification.[1,2]

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)