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Lineage-restricted Clonality in Biphasic Solid Tumors

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Cytogenetic analysis of two pulmonary chondroid hamartomas and nine breast adenofibromas revealed clonal chromosome aberrations in both hamartomas and in four breast tumors. To determine lineage of the cells with chromosome aberrations, a combined immunohistochemical/cytogenetic approach was developed that enabled simultaneous ascertainment of cytogenetic aberrations and immunohistochemical features in individual cells. Immunohistochemical/cytogenetic evaluation of one hamartoma and two adenofibromas demonstrated that neoplastic proliferation, in each case, was confined to the mesenchymal (stromal) component, whereas epithelial cells appeared to be reactive. Cytogenetically abnormal short-term cultures of the remaining hamartoma and another of the breast adenofibromas were composed entirely of mesenchymal elements, indicating mesenchymal clonality in those tumors as well. Our findings support redesignation of pulmonary chondroid hamartomas as 'pulmonary chondromas' and suggest that carcinomas developing within fibroadenomas arise from reactive epithelial proliferation. Combined immunohistochemical/cytogenetic analysis might be useful in the development of novel therapeutic approaches that selectively target neoplastic populations within solid tumors. (Am J Pathol 1991, 138:1199–1207)

Many tumors are characterized by coproliferation of epithelial and mesenchymal elements.[1] Some biphasic benign tumors, including many hamartomas and breast adenofibromas, have been regarded historically as nonclonal hyperplastic lesions, and it remains unclear whether these tumors contain neoplastic components.[2–4] Other biphasic tumors, however, are recognized as true neoplasms.[5,6] In biphasic epithelial/mesenchymal malignant tumors especially, it might be important to define the neoplastic element because mesenchymal and epithelial populations often have different natural histories and therapeutic requirements.[7]

Two biphasic tumors of controversial histogenesis are pulmonary chondroid hamartomas and breast adenofibromas. Pulmonary chondroid hamartomas have mesenchymal and epithelial components and present typically as asymptomatic coin lesions that are detected incidentally during radiographic studies or at postmortem examination.[2,3] Although thought originally to represent hyperplastic developmental remnants, several investigators suggest that the epithelial and mesenchymal components, or the mesenchymal component alone, might be neoplastic in these hamartomas.[2,3,8,9]

Breast adenofibromas are common tumors that often contain exuberant proliferation of benign mesenchymal and epithelial cells.[4,10,11] The pathogenesis of breast adenofibromas is unclear: although often attributed to nonneoplastic nodular hyperplasia,[4] other possibilities include epithelial neoplasia with reactive mesenchymal proliferation, mesenchymal neoplasia with reactive epithelial proliferation, and biphenotypic neoplasia resulting from epithelial and mesenchymal differentiation of a common progenitor. Accordingly the terms 'adenofibroma' and 'fibroadenoma' often are used interchangeably to describe these tumors. Because carcinomatous transformation sometimes occurs in breast adenofibromas,[12–14] determination of the neoplastic component is of considerable interest.

To define potential neoplastic populations in pulmonary chondroid hamartomas and breast adenofibromas, we developed a combined immunohistochemical/cytogenetic (IH/C) approach. This novel approach is based on techniques described previously for determination of lineage-restricted cytogenetic aberrations in hematologic malignancies.[15,16]

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Methods

Tissue Culture and Metaphase Preparation

A consecutive series of pulmonary chondroid hamartomas and breast adenofibromas were obtained directly from the frozen section room. All specimens were minced immediately with scalpels and then disaggregated for 4 to 24 hours in a 200 unit/ml collagenase solution (Gibco, Grand Island, NY). The disaggregated cell clusters were cultured in T25 flasks, using Roswell Park Memorial Institute (RPMI) 1640 media (Gibco) with 16% fetal calf serum, 1% 1-glutamine, 1% penicillin-streptomycin, 1% (v/v) bovine pituitary extract (Collaborative Research, Lexington, MA), and 0.5% (v/v) Mito+ Serum (Collaborative Research) in a 5% CO_2 incubator at 37°C. At least six cultures were established from each specimen. Cultures were monitored daily and the relative amounts of mesenchymal and epithelial growth were noted. Cultures from each case were harvested at staggered intervals depending on time of maximal mesenchymal and epithelial cell growth. In all cases the harvests were completed within 4 to 7 days after establishment of cultures. Harvests were accomplished through exposure of adherent cells to Colcemid (0.002 μg/ml; Gibco) for 14 hours. Cells then were released from flasks by trypsinization, treated in a 0.075 mol/l (molar) KCl hypotonic solution for 10 minutes, and fixed with two changes of 3:1 methanol:acetic acid. Slides were made by conventional techniques, using steam to assist in metaphase spreading. After 2 to 3 days of incubation on a slide warmer at 60°C, the chromosomes were banded by the G bands by trypsin using Giemsa (GTG) method.[17] At least 20 metaphases were analyzed from each tumor.

Immunohistochemical/Cytogenetic Analysis

Cytogenetically abnormal tumors that displayed both mesenchymal and epithelial growth in culture were assessed further using an alkaline phosphatase anti-alkaline phosphatase (APAAP) immunohistochemical approach. Metaphases were prepared on glass slides, as described above, and placed in a solution of TRIS buffer, pH 7.6, supplemented with 2% porcine serum. Slides then were sequentially incubated with either monoclonal antibodies to keratin proteins (AE1/AE3; Boehringer Mannheim, Indianapolis, IN) or vimentin (Dako Corp., Carpenteria, CA), followed by rabbit anti-mouse immunoglobulin antibodies (1:40 dilution; Dako Corp.) and APAAP complexes (1:50 dilution; Dako Corp.). As a negative control, a slide of each case was processed using a pan T-cell monoclonal antibody (CD2, Dako Corp.) for the initial incubation. Nonspecific binding was absent in all cases. Incubation with the primary antibody was performed for 1 hour and the subsequent incubations were performed for 40 minutes each. If further enhancement of staining was necessary, incubation with rabbit anti-mouse immunoglobulin antibodies and APAAP were repeated. At the completion of these incubations, slides were washed with TRIS buffer, placed in a freshly prepared solution containing naphthol AS-MX phosphate (6 mg/30 ml; Sigma Laboratories, St. Louis, MO) as substrate and Fast Red TR salt (30 mg/30 ml; Sigma Laboratories) as chromagen. Naphthol AS-MX phosphate was dissolved initially in dimethylformamide (6 mg in 0.6 ml) and then mixed with the buffer. The reaction mixture was prepared in 0.1 mol/l TRIS buffer, pH 8.2, which also contained levamisole (18 mg/30 ml; Sigma Laboratories) as an inhibitor of endogenous alkaline phosphatase. Slides were incubated for 20 to 30 minutes in this reaction mixture and then washed with distilled water. Anti-vimentin and anti-keratin sensitivity and specificity were established using tissue sections of a breast adenofibroma (case 9), an endometrial carcinoma, and normal tonsil. All control sections were fixed in the same manner (3:1 methanol:acetic acid) as the metaphase preparations. In each tissue section, vimentin and keratin reactivity were restricted to mesenchymal and epithelial components, respectively.

Following APAAP staining, slides were air dried, conterstained with a 0.005% solution of quinacrine mustard dihydrochloride for 10 minutes, and rinsed in running distilled deionized water for 2 minutes. Slides then were air dried and stored in the dark for 1 to 10 days (slides were stable for up to 4 months when stored in the dark). Slides were analyzed on a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, FRG) using a BP 436/8+BP excitation filter, a FT 460 chromatic beam splitter, and a LP 470 barrier filter. Under fluorescent light, quinacrine banding and APAAP Fast Red TR chromagen were assessed simultaneously in individual metaphase cells. Metaphases were photographed using Fujicolor (Fuji, Tokyo, Japan) ASA 400 color print film at automatic exposure (generally 2 to 5 seconds).

Results

Pulmonary Chondroid Hamartomas

Two pulmonary chondroid hamartomas were karyotyped (Figure 1 and Table 1). Cultures of Case 1 revealed predominantly mesenchymal growth, although approximately 10% of the cells were epithelial. Ninety five metaphases contained an unbalanced translocation: 46,XY,der (18) (18pter→18q23::12q11→12q14::12q21→12qter) (Figure 2), whereas four metaphases were diploid: 46,XY. Immunohistochemical/cytogenetic studies revealed that cells with the clonal chromosome 18 rear-

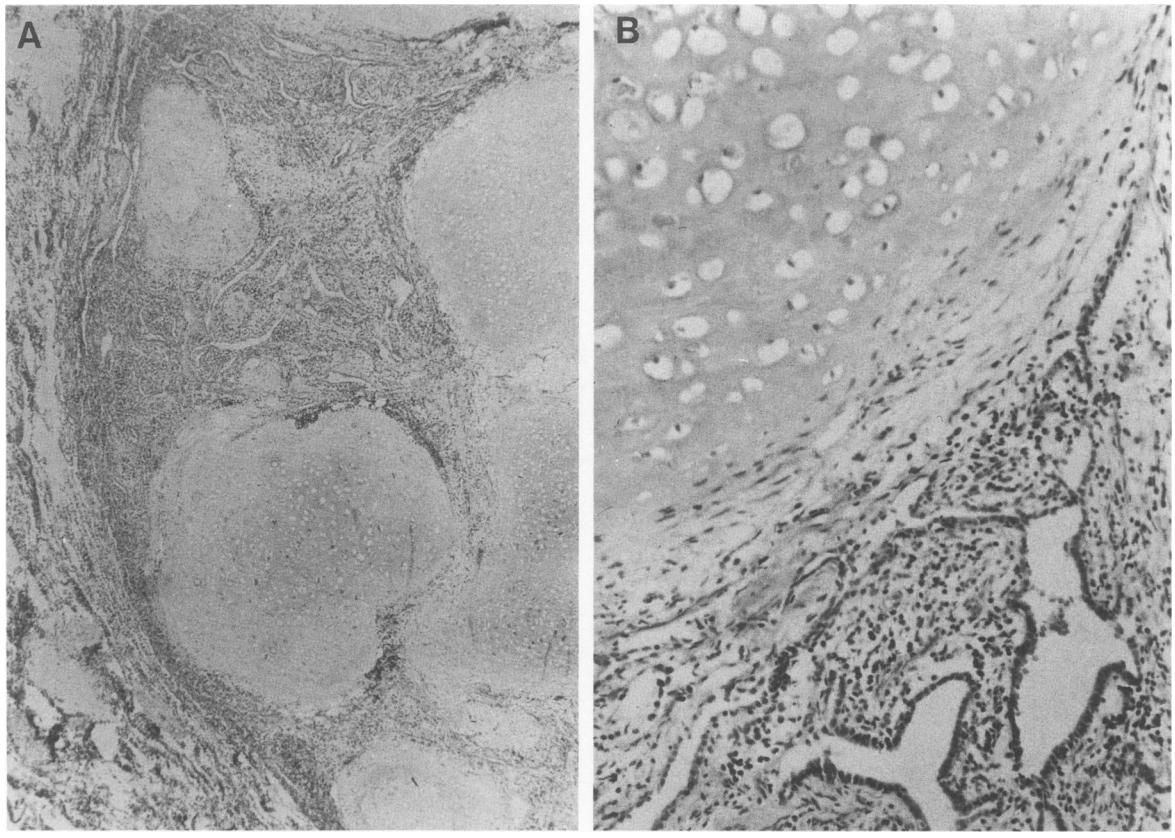

Figure 1. *Representative low-power* **(A)** *and high-power* **(B)** *views of pulmonary chondroid hamartoma case 1, demonstrating mesenchymal and epithelial components.*

rangement were uniformly vimentin positive and keratin negative (Table 2 and Figure 6). These findings confirmed that the mesenchymal population was neoplastic, whereas the epithelial proliferation appeared reactive to the neoplastic mesenchymal proliferation. Cultures of the other pulmonary hamartoma (case 2) were composed entirely of mesenchymal elements and all metaphases analyzed contained rearrangements of chromosomes 6 and 11. The cytogenetic findings indicated neoplastic clonality in the mesenchymal component of this tumor.

Table 1. *Clinical, Histopathologic, Cytogenetic, and Tissue Culture Data for Two Pulmonary Chrondroid Hamartomas and Nine Benign Breast Adenofibromas*

Case	Sex/age	Histology*	Diameter (cm)	Metaphases: Clonal abnormal karyotype	Metaphases: # Abnormal/ total	Culture morphology†
1	M/70	PCH	1.3	46,XY, −18, +der(18)(18pter → 18q23:: 12q11 → 12q14::12q21 → 12qter)	96/100	MES-EPI
2	M/64	PCH	1.5	46,XY,t(6;14)(p21;q24),del(11)(q23.2),del(11)(q23.2)	22/22	MES
3	F/34	AF	11.0	45,XX, −9, −10, −10, −19,del(6)(q21), +der(9) t(9;?)(q21;?), +der(10)t(10;?)(q21;?), +mar	11/21	MES-EPI
4a‡	F/19	AF	2.5	None	0/21	MES-EPI
4b‡	F/19	AF	1.5	None	0/14	MES-EPI
5	F/24	AF	6.0	None	0/75	MES-EPI
6	F/12	AF	5.0	53,XX, +5, +7, +12, +17, +18, +19, +20	5/20	MES-EPI
7	F/19	AF	4.0	None	0/46	MES-EPI
8	F/17	AF	7.5	None	0/20	MES-EPI
9	F/39	AF	8.0	47,XX, +11	16/22	MES-EPI
10	F/46	AF	7.5	52,X, −X, +5, +11, +20,del(1)(q11), del(2)(q31), +2r(7), +mar1, +mar2	3/13	MES

* PCH, pulmonary chondroid hamartoma, AF, adenofibroma.
† MES, mesenchymal, EPI, epithelial.
‡ 4a and 4b were two separate adenofibromas excised from the same breast.

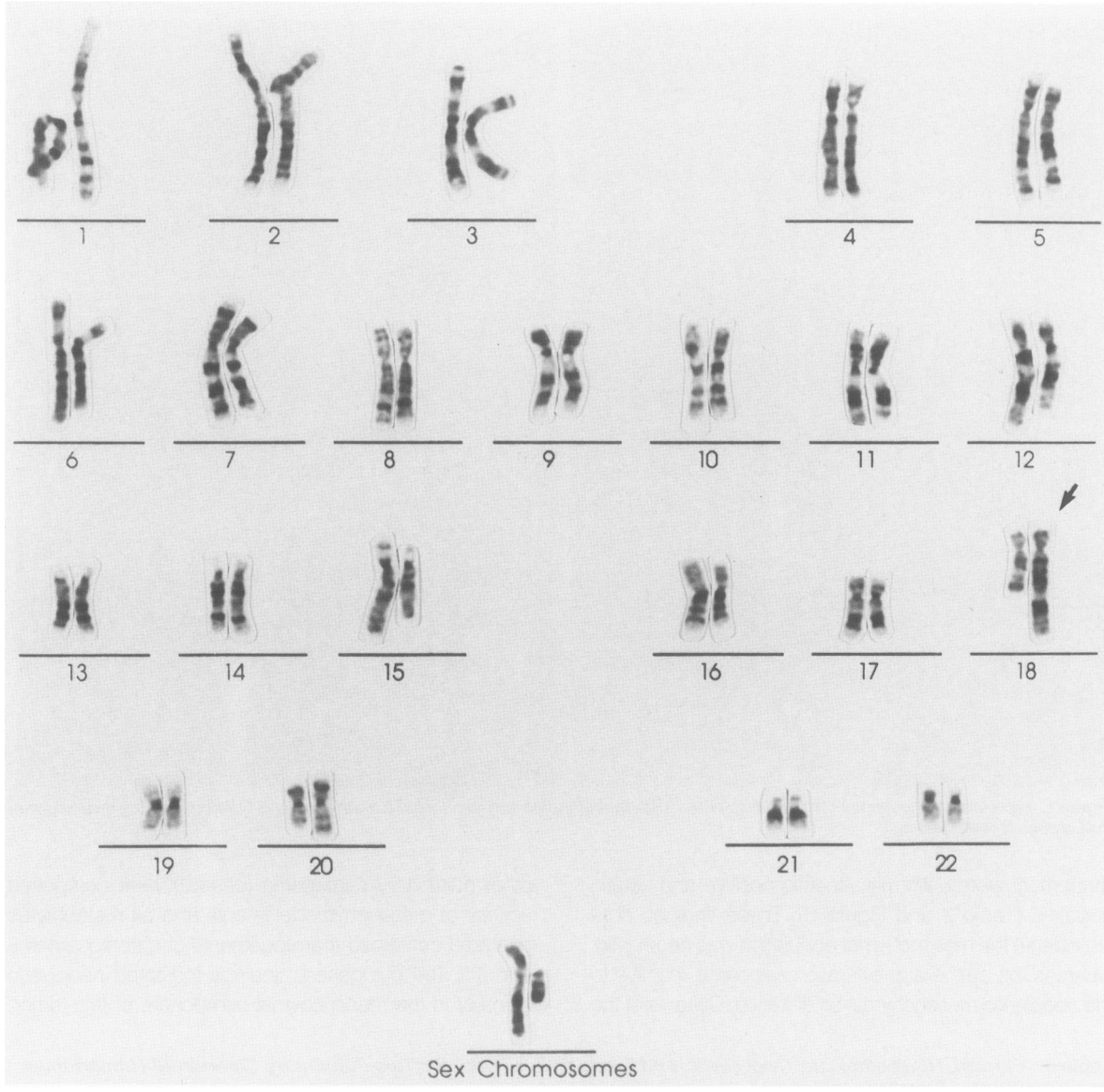

Figure 2. *GTG-banded karyotype of pulmonary chondroid hamartoma case 1 demonstrating the chromosome 18 rearrangement (arrow) that was found in most cells cultured from this tumor.*

However, because the epithelial component failed to grow in culture, IH/C studies were not performed.

Breast Adenofibromas

Nine breast adenofibromas were karyotyped (Figure 3 and Table 1). Four adenofibromas (cases 3, 6, 9, and 10) were mosaics containing cells with clonal cytogenetic aberrations and other cells that lacked chromosome aberrations. The remaining five tumors lacked apparent chromosome aberrations. Adenofibromas with clonal chromosome aberrations were similar histologically to those that lacked such aberrations. All tumors with clonal chromosome aberrations, but only two of those lacking aberrations, were at least 5 cm in maximum diameter. One cytogenetically abnormal tumor (case 6) had insufficient tumor metaphases for IH/C analysis and short-term cultures of another (case 10) were composed entirely of mesenchymal elements. Cultures of the remaining two cytogenetically abnormal tumors (Figures 4 and 5) contained exuberant mesenchymal and epithelial proliferation, with neither population predominating. These two cases were studied further using the IH/C approach (Table 2 and Figure 6). In both cases, cells with clonal chromosome aberrations were uniformly vimentin positive and keratin negative. These findings demonstrate that mesenchymal proliferation represented the neoplastic proliferation in both tumors. Cytogenetically normal cells were found in both keratin-positive and vimentin-positive

Table 2. *Combination Immunohistochemical/Cytogenetic Studies: Percentage of Cells with Clonal Cytogenetic Aberrations in Each Immunohistochemical Category*

	Percentage of Cells with Clonal Aberrations			
	Vimentin		Keratin	
Tumor	Positive	Negative	Positive	Negative
Case 1 (PCH)*	100% (43/43)†	0% (0/1)	0% (0/2)	100% (15/15)
Case 3 (AF)*	61% (22/36)	0% (0/21)	0% (0/14)	85% (17/20)
Case 9 (AF)	88% (14/16)	0% (0/5)	0% (0/8)	90% (28/31)

* PCH, pulmonary chondroid hamartoma, AF, adenofibroma.
† No. of cells with clonal aberrations/total cells analyzed in this category.

categories, indicating that the entire epithelial population and a component of the mesenchymal population were reactive proliferations.

Discussion

Orderly stromal–epithelial interaction is essential in the normal development[18] and repair[19] of many organs, and the same interactions presumably contribute to the formation of certain benign and malignant solid tumors. The mechanisms of these interactions are poorly understood,[20] but it is known that fibroblasts can accelerate the growth of epithelial cells.[21] Specific growth factors, including keratinocyte growth factor,[22] may be responsible for this fibroblast–epithelial interaction. In the present study, combined IH/C characterization of one pulmonary chondroid hamartoma and two breast adenofibromas demonstrated a clonal neoplastic mesenchymal component in each tumor. The epithelial population appeared to be reactive in each tumor and substantial subgroups of the mesenchymal cells were reactive in the two breast tumors.

This report, to the best of our knowledge, is the first demonstration of clonality in so-called pulmonary hama-

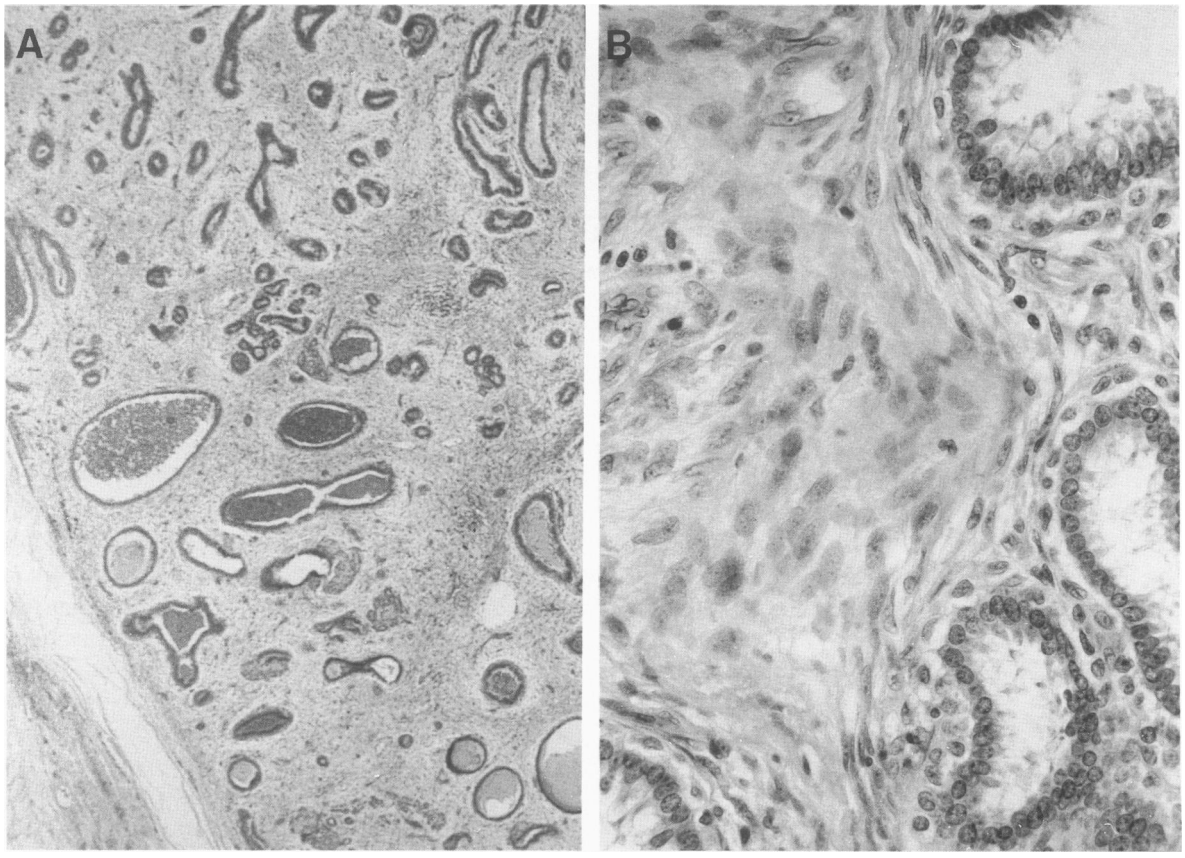

Figure 3. *Representative low-power* **(A)** *and high-power* **(B)** *views of breast adenofibroma case 3 demonstrating mesenchymal and epithelial components.*

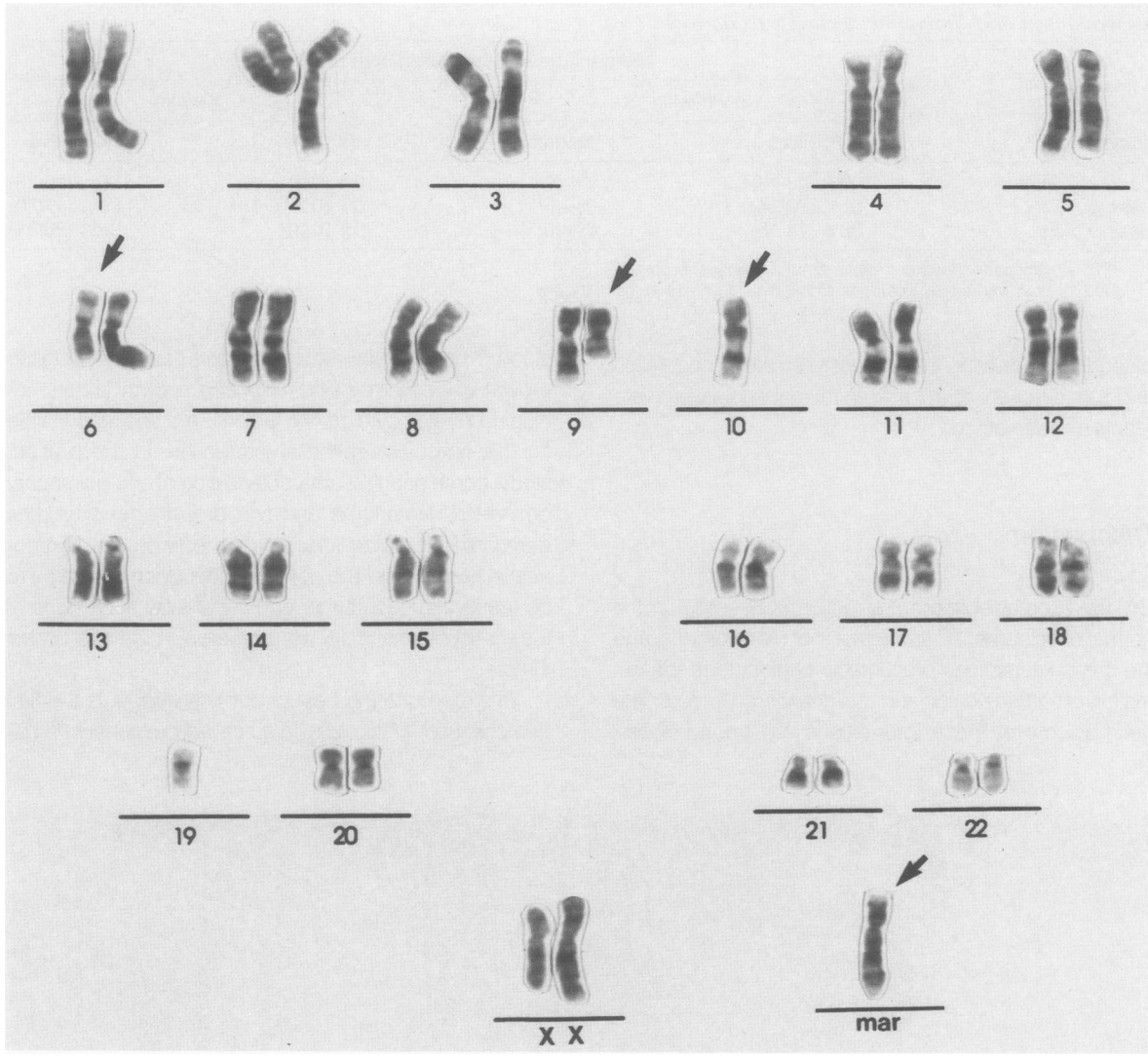

Figure 4. *GTG-banded karyotype of adenofibroma case 3 demonstrating multiple clonal chromosome aberrations (arrows) that were found in approximately 50% of cells cultured from this tumor.*

rtomas. Other investigators postulated a neoplastic component in lung hamartomas,[3] and the cytogenetic aberrations described herein confirm that suspicion. However the different cytogenetic events in our two cases indicate some degree of genetic heterogeneity in the development of these unusual neoplasms. Because the term 'hamartoma' generally connotes a nonneoplastic proliferation, the present evidence support redesignation of PCH as 'pulmonary chondroid mesenchymomas' or 'pulmonary chondromas.'

Previously an extra chromosome was demonstrated in one half the cells from one breast adenofibroma[23]; however, in the absence of modern banding techniques, that chromosome could not be identified with certainty. Four of nine adenofibromas in the present series had clonal cytogenetic aberrations, but none of the aberrations were shared by more than two of the tumors. Accordingly our data indicate substantial cytogenetic heterogeneity, which presumably reflects alternate mechanisms of mesenchymal transformation in different adenofibromas. Because the mesenchymal component was clearly neoplastic in three tumors (cases 3, 9, and 10), these specific neoplasms would more properly be designated 'adenofibromas' rather than 'fibroadenomas.' Although not demonstrated in this study, it is possible that epithelial proliferation is the neoplastic component in other breast adenofibromas. It should also be noted that all cytogenetically abnormal adenofibromas were relatively large tumors (Table 1) and it is unclear whether most small (less than 2 cm) adenofibromas represent primary mesenchymal proliferations. Based on our preliminary findings, however, it appears likely that carcinomatous transformation in some breast adenofibromas[12–14] occurs in cells that originate from reactive epithelial proliferation.

Breast adenofibromas are most common in young

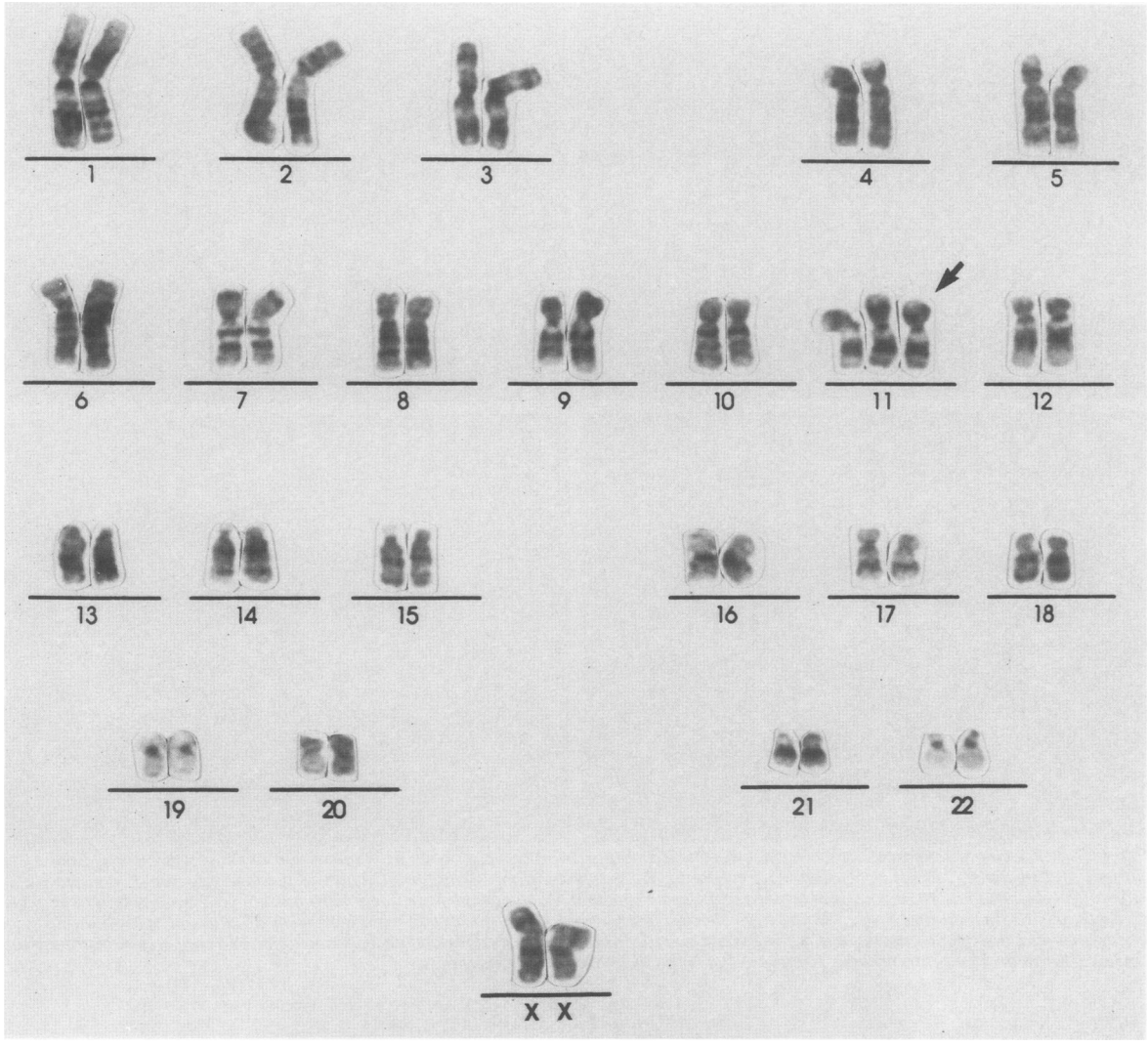

Figure 5. *GTG-banded karyotype of adenofibroma case 9 demonstrating trisomy 11 (arrow), which was found in more than 50% of cells cultured from this tumor.*

women[4] and are presumed to result, in part, from hormonal stimuli. Accordingly the primary nature of mesenchymal proliferation in our adenofibromas suggests that breast stroma, like breast epithelia, is responsive to estrogen and/or progesterone. Estrogen and progesterone receptors have been observed in many mesenchymal malignancies[24] and also have been noted, recently, in the mesenchymal component of a vaginal fibroepithelial polyp.[25] In addition, elevated levels of progesterone and estrogen receptors have been detected by biochemical assay in breast adenofibromas.[26] These observations suggest that hormonal treatment of some benign and malignant breast tumors might suppress both epithelial and mesenchymal elements in those tumors.

Our combined IH/C approach is related to techniques previously used to assess cell lineage in hematologic neoplasia.[15–16,27] Whereas chromosome and immunohistochemical profiles were assessed sequentially in the earlier approaches, the present methodology allows simultaneous determination of chromosome banding and immunohistochemical characteristics. Simultaneous fluorescent IH/C analysis enables delineation of lineage-restricted chromosome aberrations in an extremely sensitive and efficient manner. This methodology can be applied readily to any cytogenetically abnormal mixed-lineage proliferation as long as the primary immunohistochemical antibody binds appropriately to determinants that have been fixed with the methanol:acetic acid solution used for metaphase preparations. In the present report we demonstrated that keratin and vimentin detection is not hampered by methanol:acetic acid chromosome fixation. Desmin also can be demonstrated reliably after such fixation, whereas neuron-specific enolase is not recognized (Fletcher JA, Pinkus GS, unpublished data). One limitation of past and present IH/C methods is the requirement for dividing cells, obtained from direct harvests of

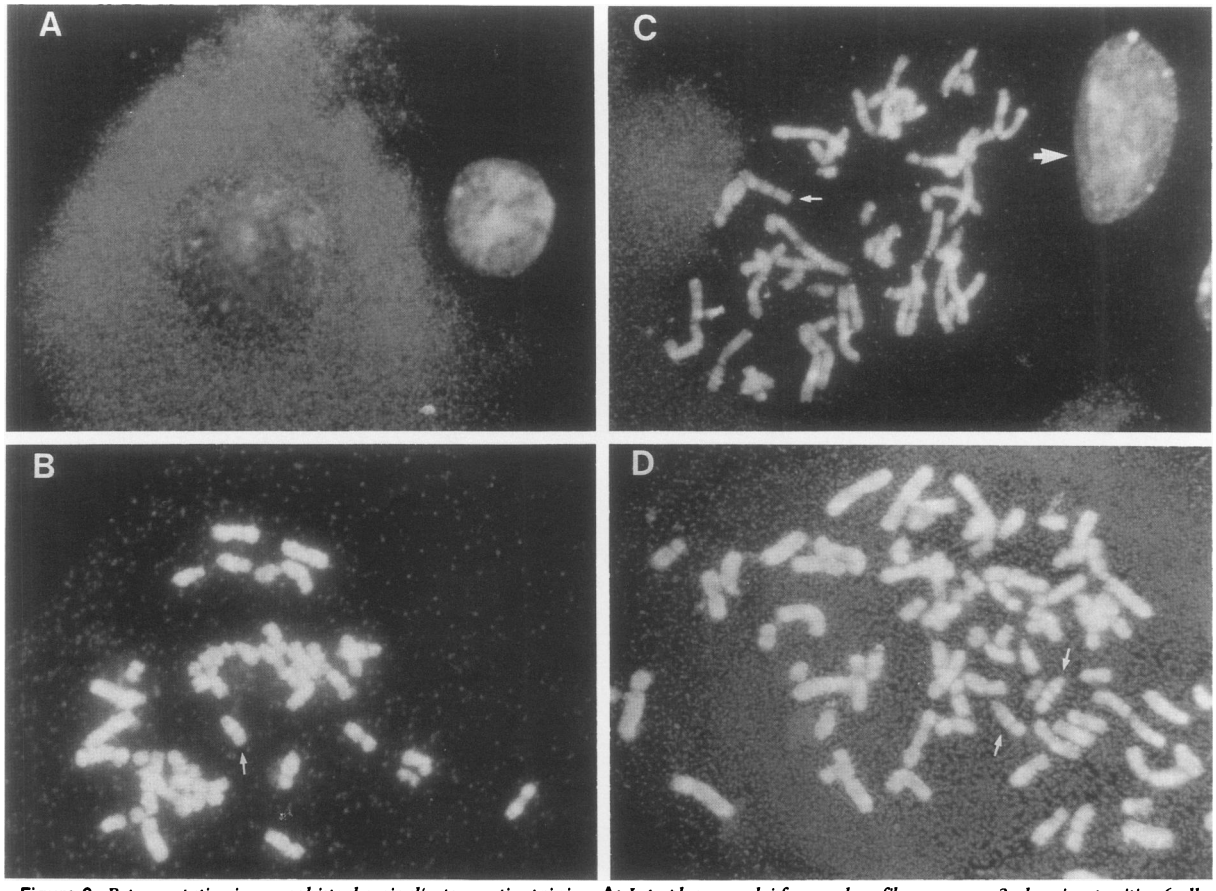

Figure 6. *Representative immunohistochemical/cytogenetic staining.* **A:** *Interphase nuclei from adenofibroma case 3, showing positive (cell on left) and negative (cell on right) staining for keratin.* **B:** *Metaphase from pulmonary chondroid hamartoma case 1, demonstrating rearranged chromosome 18 (arrow) and positive staining for vimentin.* **C:** *Metaphase from adenofibroma case 3, demonstrating rearranged chromosome 10 (arrow) and lack of staining for keratin. Peripheral keratin staining is from cytoplasm of adjacent epithelial cells. One keratin-negative interphase nucleus (arrowhead) is also seen.* **D:** *Endoreduplicated metaphase from adenofibroma case 3, demonstrating two copies of the rearranged chromosome 10 (arrows) and positive staining for vimentin.*

high-grade tumors or from tissue culture, that provide metaphases for cytogenetic analysis. Future development of combined fluorescent *in situ* hybridization/immunohistochemical detection methods will permit detection of lineage-restricted chromosome aberrations in interphase cells. These methods will facilitate analysis of low-grade solid tumors without the need for tissue culture.

As consistent genetic aberrations are identified increasingly in solid tumors,[28] the IH/C approach should be extremely helpful in characterizing lineage-restricted clonality within those tumors. In many cases, such determinations might have biologic relevance. For example, shared genetic aberrations in multiple cell types within a given tumor would implicate a stem cell defect, whereas lineage-restricted aberrations would suggest transformation of more differentiated cells. This information might be crucial in devising and evaluating novel antineoplastic approaches, eg, monoclonal antibody-toxin conjugates or retroviral vectors, which target specific cellular phenotypic determinants.

Acknowledgments

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