Papers

Atypical ductal hyperplasia of the breast: clonal proliferation with loss of heterozygosity on chromosomes 16q and 17p

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Abstract

Aims—To determine if allelic loss on chromosomes 16q and 17p, commonly encountered in in situ and invasive ductal carcinomas, is present in atypical ductal hyperplasia (ADH); to determine whether ADH is a neoplastic (clonal) or hyperplastic (polyclonal) proliferation.

Methods—Fourteen cases of ADH were examined for allele loss at loci on chromosome 16q and 17p using a microdissection technique, polymorphic DNA markers and the polymerase chain reaction (PCR).

Results—Loss of heterozygosity (LOH) was detected in five of nine informative cases on chromosome 16q at the microsatellite D16S413 and two of eight informative cases on chromosome 17p at D17S796.

Conclusions—The incidence of LOH at these loci is similar to that previously observed in ductal carcinoma in situ and in invasive ductal carcinoma. Because of the nature of the technique used, our findings also demonstrate that ADH is a monoclonal, and hence, neoplastic proliferation rather than a hyperplastic (polyclonal) condition as its name suggests. There is thus a case for including ADH, as presently defined, within the spectrum of ductal carcinoma in situ.

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exhibit some, but not all, features of ductal carcinoma in situ.^{4,5} To address this problem, Page and Rogers⁶ laid down clear criteria for diagnosing ADH based on combined histological and cytological features. Despite the use of these strict criteria, a recent study in which cases of hyperplasia of usual type (HUT), ADH and ductal carcinoma in situ were examined by six experienced breast pathologists showed complete agreement in only 58% of cases.⁷ Given the similarity of ADH to ductal carcinoma in situ of low nuclear grade, it would clearly be of value to learn more about these processes at the molecular level, particularly if such investigations led ultimately to the development of more objective diagnostic criteria.

We have recently developed methodology for detecting allele loss in microscopic lesions dissected from formalin fixed, paraffin wax embedded tissue using the polymerase chain reaction (PCR). Using this technique, we have found loss of heterozygosity (LOH) at loci on chromosomes 16q and 17p in approximately 50% of cases of ductal carcinoma in situ.⁸ LOH can be detected by this method only when the relevant allele is lost from virtually all of the cells under study—that is, when they are monoclonal, as loss of an allele is unlikely to occur in all cells independently.

The aims of this study were to determine whether allelic loss on chromosomes 16q and 17p is present in ADH, and to determine whether ADH is a neoplastic (clonal) or hyperplastic (polyclonal) proliferation.

Methods

Cases of ADH were retrieved from the files of the Royal Marsden NHS Trust. Fourteen fulfilled the criteria for ADH as defined by Page and Rogers,⁶ showing partial involvement of membrane bound spaces by a monomorphic population of cells with small hyperchromatic nuclei similar to those seen in the low nuclear grade variants of ductal carcinoma in situ. Although some of the secondary lumina were “punched out”, others were irregular and ill defined. Cell bridges often showed a tapered appearance and a layer of polarised cells was identified at the periphery of basement mem-

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Details of the 10 informative cases for loci on chromosomes 16q and 17p

Case No.	Age (years)	Size of lesion (mm)	Extent of lesion ⁷	Associated lesions		LOH	
				Benign	Malignant	16q (D16S413)	17p (D17S796)
207	64	2.0	M	Cysts	DCIS-(ING-Pap) + IDC gd II	+	+
246	86	4.5	F	Papilloma	DCIS-(HNG-Pap) + IDC Pap type	+	—
251	58	1.0	E	Radial scar, hyperplasia of usual type	—	+	+
		1.0				+	+
		1.0				+	+
259	55	2.0	F	Radial scar, hyperplasia of usual type	—	—	ni
		2.0				+	ni
261	46	3.8	F	Cysts	—	+	pu
		—				+	—
197	56	1.6	M	Hyperplasia of usual type	DCIS-(HNG-Comedo) + Microinvasion	ni	—
		1.7				ni	—
205	57	0.4	F	Hyperplasia of usual type, sclerosing adenosis, papilloma	—	—	—
232	65	0.5	F	—	LCIS + ILC	—	—
247	49	2.0	F	Hyperplasia of usual type, sclerosing adenosis	DCIS-(HNG-Solid) + IDC gd II	—	—
		1.7				—	—

DCIS = ductal carcinoma in situ; IDC = invasive ductal carcinoma; LCIS = lobular carcinoma in situ; ILC = invasive lobular carcinoma; Pap = papillary; gd = grade; ING = intermediate nuclear grade; HNG = high nuclear grade; F = focal; M = moderate; E = extensive; ni = not informative; pu = PCR unsatisfactory.

brane bound space. Hence, they showed some, but not all, features of low grade ductal carcinoma in situ. The lesions were measured using the micrometer on the slide stage of the microscope. The extent of the lesion was assessed according to the criteria laid down by Tavassoli and Norris.⁹

Six of the 14 cases were associated with in situ or invasive carcinoma and eight with only benign changes (table). Uninvolved lymph node or normal breast tissue was used to provide constitutional DNA from each patient.

DISSECTION OF NEOPLASTIC TISSUE

Three 15 µm paraffin wax sections were cut from each case onto double-sided clear adhesive tape and placed upon glass slides. They were stained lightly with toluidine blue to highlight the relevant areas. Under a dissecting microscope, a fine scalpel blade was used to cut around the area of intraductal proliferation and the same area from the three slides was peeled off and placed into a 1.5 ml Eppendorf tube. Sections (5 µm) taken immediately before and after the 15 µm sections were stained with

Figure 1 Case 207. **A:** The area delineated by the arrows was dissected. The proliferation consists of cells with small rounded (but not particularly hyperchromatic) nuclei and exhibiting a cribriform growth pattern. Nuclear placement is even in some areas but not in others. Some secondary lumina are well demarcated whereas others are indistinct. Some rigid as well as many non-rigid tapering cellular bars are seen and columnar polarised cells are identified above the basement membrane, especially on the right of the picture. This was the most florid example included in the study. (Haematoxylin and eosin; magnification $\times 400$.) **B:** LOH at 16q and 17p is seen in this focus of ADH. A focus of intermediate cell micropapillary ductal carcinoma in situ found elsewhere in the breast was also examined and also showed LOH at both loci. This lesion was morphologically different from the focus of ADH. Each allele is arrowed. There are a series of “stutter” bands due to imperfect “reading” of the microsatellites by PCR. DCIS = ductal carcinoma in situ; LN = lymph node.

Figure 2 Case 251. *A: The arrows identify the lesion that was dissected. The cells have similar morphology to those seen in fig 1 and the growth pattern is cribriform and micropapillary. The papillary processes often contain thin fibrovascular cores. Nuclear placement is uneven and there is a row of polarised cells around the periphery. (Haematoxylin and eosin; magnification $\times 400$.) B: This and two similar lesions were dissected, all showing allelic loss on 16q and 17p. LN = lymph node.*

haematoxylin and eosin. These sections were used to identify the relevant lesions and to estimate the level of contamination by normal stromal and inflammatory cells. The proportion of normal to tumour cells was estimated using an image analysis system.

DNA EXTRACTION

Dissected fragments were incubated for 16 hours at 37°C in 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1% (w/v) sodium dodecyl sulphate and 500 µg/ml proteinase K. The mixture was then heated to 100°C for 10 minutes to inactivate the proteinase K and aliquots were used directly for PCR.

ANALYSIS OF POLYMORPHIC DINUCLEOTIDE REPEATS

To analyse loci on chromosomes 16q and 17p, aliquots of the digested material were amplified using PCR in 50 µl of 50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% (v/v) Triton X-100, 2.5 mM MgCl₂, 0.5 M Taq polymerase, 0.1% (w/v) bovine serum albumin, 2 mM each dNTP, and each primer at 5 ng/µl. In a typical experiment, 35 cycles of 94°C for one minute, 55°C for one minute and 72°C for one minute were carried out. One primer of the pair for each locus was 5' end-labelled using γ [³²P] ATP and T4 polynucleotide kinase. The amplified products were electrophoresed through 6% denaturing polyacrylamide gels and exposed to x

ray film for 16 hours to five days. Cases showing evidence of allele loss were repeated at least once to confirm the loss. Allele loss was also confirmed by scanning densitometry or by exposure on a phosphorimager.

Two dinucleotide repeats, D16S413 and D17S796, that lie within genomic regions which exhibit LOH in invasive and in situ ductal carcinoma were examined. D16S413 is located on chromosome 16q and D17S796 on 17p within 2 cM of the p53 gene (Easton D, personal communication, 1994).

Results

Of the 14 cases studied, 10 were informative—that is, heterozygous, for at least one marker and clear bands representing the two alleles were seen in control samples of normal breast tissue or lymph nodes. Suspected allele loss within the ADH sample was confirmed by comparing the relative intensity of the tumour and control bands using a densitometer or phosphorimager. The details of these 10 cases are shown in the table. Nine of 10 cases were informative each for D16S413 and D17S796. In one case studied for D17S796, however, technical problems were encountered with the PCR. Interpretable results were thus obtained at this locus in only eight cases. In the remaining four cases, it was not possible to interpret the results of the PCR with either marker. Only two loci were studied as ADH tends to be small and hence the amount of DNA available from any one focus is limited.

Of the 10 informative cases, five showed LOH. All five showed at least one focus with LOH at 16q and two of these cases also had loss of 17p (figs 1 and 2). Two of five cases exhibiting LOH were associated with in situ and invasive carcinoma while the other three cases had benign changes only, including cysts, HUT and radial scars. As can be seen from the table, the sizes of the lesions in cases exhibiting LOH range from 1.0 to 4.5 mm and the disease extent from focal to extensive. The age range was 46–86 years (mean 61.8 years).

More than one separate focus of ADH was examined from the same breast in three of five informative cases. Identical results were obtained with each focus from any particular case with the exception of 259 where LOH on 16q was found in one focus of ADH but not in the other.

The five cases which did not show LOH in any sample at either locus had a similar age distribution ranging from 49 to 65 years (mean 57.7 years). Three cases had associated in situ and invasive carcinoma, one had benign changes and the fifth was otherwise normal. All lesions were small ranging from 0.4 to 2.0 mm and four of five were focal.

We did not detect any significant morphological differences between the cases with and without LOH. In all the cases the estimated level of contamination by stromal or inflammatory cells did not exceed 5%.

Some of the cases of ADH had adjacent in situ and invasive carcinoma. Data on LOH in these lesions are not presented here as they are not deemed to be relevant to the present study, especially as allelic loss in ADH has also been identified in otherwise benign breast biopsy specimens.

Discussion

Using a microdissection technique to isolate small foci of ADH and by amplifying polymorphic dinucleotide repeats (microsatellites) using PCR, we have shown that half of our cases of ADH, as defined by Page and Rogers,⁶ were monoclonal proliferations exhibiting allelic loss at loci on chromosomes 16q and 17p. It is likely that the other half were also monoclonal given that they were morphologically identical; however, they lacked detectable deletions at the precise loci studied. The incidence of LOH is similar to that already found in ductal carcinoma in situ of high and low nuclear grade⁸ and which has been found in invasive ductal carcinoma by other authors using Southern analysis.¹⁰ That the detection of LOH in ADH was due to extension of nearby carcinoma cells is excluded by the lack of malignancy in three cases and the cytological dissimilarity of the in situ and invasive malignancy in the other two cases (table). Where more than one focus was examined from the same case, the pattern of LOH was the same with one exception where two foci differed with respect to the 16q deletion. Whether this reflects aspects of methodology or genuine subclonal heterogeneity is not yet clear. The former is possible,

particularly as the gels were reported very conservatively and LOH was recorded only when a very obvious result was obtained.

It is possible to determine allelic loss using the method we have described only when the sequence amplified by PCR is lost from the great majority of cells in the proliferation. Where significant numbers of cells have retained the relevant allele, the PCR will amplify it sufficiently to produce a second band and hence obscure the deletion that would otherwise be identified. The most likely explanation for the presence of allelic loss in a cell population is that they are all descendants of a single cell in which the genetic lesion first occurred. The alternative explanation that all the cells acquired an identical genetic lesion independently is highly improbable. Consequently, our findings indicate that ADH is a monoclonal and, therefore, neoplastic proliferation rather than a hyperplastic (polyclonal) proliferation as its name suggests.

Further evidence for the clonal nature of ADH is provided by a recent study in which it was found to be monoclonal with respect to X chromosome inactivation.¹¹ A potential problem with this method is that the "patch" size with respect to X inactivation in the human breast has not, to the authors' knowledge, been determined. If it is large and includes whole lobules, then proliferations could arise from a large number of cells but still appear clonal. In addition, these authors did not clearly define their criteria for the diagnosis of ADH. Nevertheless, their study and the present one together provide strong evidence that ADH is a neoplastic process exhibiting at least some of the genetic changes which characterise established in situ and invasive breast carcinoma. There is thus a case for including ADH, as defined by Page and Rogers,⁶ within the spectrum of ductal carcinoma in situ, either using existing terminology or less specific nomenclature such as ductal in situ neoplasia.

It has become recognised in recent years that ductal carcinoma in situ is a heterogeneous group of proliferations in which the histological variants exhibit different clinical behaviour.¹² Subclassification of ductal carcinoma in situ has thus become a widely accepted necessity. To what extent ADH and low nuclear grade forms of ductal carcinoma in situ differ in their clinical behaviour requires further investigation. As the major problem of diagnosing ADH lies in distinguishing it from ductal carcinoma in situ of low nuclear grade,^{4,5} the inclusion of ADH within the spectrum of ductal carcinoma in situ might improve diagnostic consistency.

The finding of 16q and 17p deletions in ADH indicates that they are early events in the development of invasive cancer. We are undertaking further studies of a wide range of proliferative lesions to determine at which stage in the morphological evolution of breast cancer they are first detectable.

We have previously undertaken similar studies in lobular carcinoma in situ using the same technique. Allele loss on chromosome 16q was found with similar frequency to that seen in

ductal carcinoma in situ and ADH but there was a reduced incidence of 17p deletion (approximately 8%).¹³ The approximately similar incidence of 16q deletions at the locus studied suggests that lobular carcinoma in situ, ductal carcinoma in situ and ADH may share a common evolutionary pathway.

Molecular genetic investigations of the type undertaken in this study may well help in future classifications of borderline breast lesions but further work is clearly required. The incidence of LOH at the chromosomes studied may have been underestimated as so far we have been able to study only one marker on each chromosome. Furthermore, loci on other chromosomes may provide suitable markers for study of early breast cancer. Our preliminary studies have not revealed any correlation between allelic loss on chromosomes 16q and 17p and any of the currently available clinical or pathological data, but this is worth further investigation, particularly to determine whether specific deletions have prognostic significance within this group of borderline lesions.

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