Clonality analysis of intraductal proliferative lesions using the human androgen receptor assay

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Received January 8, 2007

Recently, it is accepted that invasive breast carcinoma is of monoclonal origin. Ductal intraepithelial neoplasia (DIN) may progress toward invasive carcinoma with an increased risk. However, it is not fully understood whether DIN is polyclonal or monoclonal. In this current study, we detected clonal origin of DIN using x-inactivation at the human androgen receptor (HUMARA) locus. Lesional and normal breast gland cells were microdissected from paraffin-embedded tissues using a laser capture microdissection system. Genomic DNA was extracted. After digestion by restriction enzyme Hpa II, the HUMARA exon1 was amplified by a fluorescent nested-PCR procedure and the PCR products were separated on DNA sequencer and analyzed the fluorescent intensity of the two HUMARA alleles. DNA from 88 of 101(87%) patients was able to be amplified at the HUMARA locus and 68 of them (77.3%) were heterozygous and informative. 9/12 usual ductal hyperplasia (UDH) and 5/18 DIN 1A showed a polyclonal inactivation. 3/12 UDH, 13/18 DIN 1A, 28/28 DIN 1B, 10/10 carcinoma in situ are of monoclonal origin. Taken together, DIN 1A, 1B and carcinoma in situ, are monoclonal and DIN 1, but not UDH, represents the obligate and direct precursor of DCIS.

Key Words: breast; intraductal proliferative lesions; laser capture microdissection; HUMARA assay; X-inactivation

Intraductal proliferative lesions are a group of cytologically and structurally diverse types of proliferations, typically originating from the terminal duct-lobular unit and confined to the mammary duct lobular system. They can be categorized generally as usual ductal hyperplasia (UDH), ductal intraepithelial neoplasia, including grade1A (flat epithelial atypia), 1B (atypical ductal hyperplasia, ADH), 1C (ductal carcinoma in situ), grade 2(ductal carcinoma in situ, intermediate grade), grade 3(ductal carcinoma in situ, high grade) [1].

Clinical follow-up studies have indicated that they are associated with an increased risk, albeit of greatly different magnitudes, for the subsequent development of invasive carcinoma [2]. A step-wise increase in cancer risk, as suggested by epidemiological data and morphological findings, led many investigators to favor the view of a biological continuum or progression cascade starting at UDH and ending at overtly invasive breast cancer [3–8]. Population–based mammography screening has resulted in increased detection of intraductal proliferative lesions. These raise the question as to where be the turning point in this progression cascade. That is to say, what type of intraductal proliferative lesions is already neoplasia?

It is well known that most neoplasms are of monoclonal origin [9, 10]. A better understanding of clonal origin of intraductal proliferative lesions should shed new light on the initiation and progression of breast carcinoma.

Lyon [11] showed that the random inactivation of either the paternal or the maternal X chromosome occurs by methylation during early embryogenesis of females and the progenitor cells stably inherited the methylation status. This leads to somatic mosaicism of normal females with respect to X-linked alleles, with approximately one-half of the somatic cells expressing the maternal allele and the other half expressing the paternal allele. Tumors arising from a single cell will therefore express one of the two phenotypes. The human an-

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Abbreviations: atypical ductal hyperplasia (ADH), cytokeratin (CK), corrected allele ratio (CR), ductal carcinoma in situ (DCIS), dutal intraepithelial neoplasia (DIN), human androgen receptor (HUMARA), smooth muscle actin (SMA), usual ductal hyperplasia (UDH)

drogen receptor (HUMARA), which is located on the X chromosome, has a highly polymorphic (approximately 90%) CAG repeat and methylation-sensitive restriction enzyme sites of HpaII and HhaI. [12–14]. Currently, it has been frequently used to distinguish the two alleles and to detect monoclonality in various neoplastic diseases, including acute myeloid leukemia (14), kaposi's sarcoma [15], sporadic angiomyolipoma [16], and breast carcinoma [17].

In this study, we used HUMARA gene analysis to examine the clonality of intraductal proliferative lesions. The results showed that DIN1A, 1B and carcinoma in situ, are monoclonal and DIN 1, not UDH, represents the obligate and direct precursor of DCIS, which may contribute to better understanding of how breast carcinoma originated and even to clinical management of DIN 1A, 1B.

Materials and methods

Tissue specimens. 101 Breast specimens were obtained from female patients who underwent surgery for palpable mass at Huashan hospital, between Jan 2004 and June 2006. Their mean age was 52.67 years (rang, 31 to 80). The clinical characteristics of the patients are summarized in Table 1. Normal tissue samples were obtained from the edge of lesions. All experiments were performed according to the guidelines of the ethics committee.

According to the criteria of World Health Organization Classification of Tumors, the lesions were diagnosed and classified on routine haematoxylin and eosin (H&E)-stained sections by two experienced pathologists. Immunohistochemistry for cytokeratin 5/6(CK5/6) was used as a diagnostic adjunct to distinguish UDH from DIN 1A. All together 12 cases of UDH, 18 cases of DIN 1A, 28 cases of DIN 1B, 10 cases of carcinoma in situ were investigated.

Microdissection and DNA extraction. Paraffin-embedded blocks of formalin–fixed tissues were sectioned to a thickness of 6 μm and the sections were lightly counterstained with hematoxylin. Lesions of DIN1A and UDH are very small. To avoid contaminating myoepithelial cells on Lase capture microdissection (LCM), immunohistochemical staining of smooth muscle actin (SMA) was performed to label myoepithelial cells (as described below). Target epithelial cells were procured using Veritas™ microdissection instrument (Arcturus Bioscience, Mountain View, CA). The average area of cells captured on each cap from an individual lesion and from normal breast tissue was approximately $1mm^2$ and 5 $000\mu m^2$, respectively. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, USA) and the DNA concentration was determined using BioPhotometer (Eppendorf, German). Both procedures were performed following manufacturers instructions.

Immunohistochemical staining for SMA and CK5/6. Deparaffinized sections were pretreated in citric acid buffer at 95°C for 20 min. Then quench endogenous peroxidase in 1% H_2O_2 in methanol for 20 min. Sections were incubated for 1 hour with primary monoclonal mouse antibody at 1:100 dilutions (anti-SMA: PROGEN, ASM-1 and anti-CK5/6: Zymed, D5/16B4), followed by incubation with Anti-mouse-HRP Superenvison™ (Dako, Santa Barbara, CA, USA)for 45 min. The reaction products were visualized by 3, 3'-diaminobenzidine (DAB) (Sigma Chemical, StLouis, MO, USA), and the sections were then lightly counterstained with hematoxylin. Controls were incubated with mouse IgG at the same concentration as the primary antibody.

Table 1 Summary of specimens' characteristics and results of colonal analysis

Diagnosis	UDH	DIN1A	DIN1B	DCIS	total
Monoclonal	3	13	28	10	54
Polyclonal	9	5	0	0	14
Total	12	18	28	10	68

Clonality assessment. Each DNA sample (1μg) was digested with 5U HpaII (Invitrogen, Catalog Number – 15209-018) overnight at 37°C and then inactivated by heating at 95°C for 10 min. A control reaction was set up under the same conditions but without HpaII. Exon 1 of the HUMARA was amplified from 2μl digested and undigested DNA by a nested PCR using the GC-rich PCR System (Roche Diagnostics, Indianapolis, IN) performed as previously described [18]. After amplification, PCR products were electrophoresed on an ABI PRISMTM 310 Genetic Analyzer (Applied Biosystems), and then were analyzed for fluorescent intensity of the two HUMARA alleles using Genescan 311 and Genetyper 3.7 Software (Applied Biosystems), with the allele peak heights serving as a semi quantitative measure of the amount of PCR products.

Data interpretation. To correct possible preferential amplification of one allele, a corrected allele ratio (CR) was calculated. CR was derived by dividing the ratio of the undigested allele1 and the digested allele1 by the ratio of the undigested allele2 and the digested allele2. We defined samples as monoclonal if CR was less than 0.33 or above 3 [19]. Polyclonal tissues with random X-inactivation patterns would be expected to have ratios equal or close to 1.0.

Statistical analysis. Differences in incidence of monoclonality were compared between each histological group and calculated by using the Fisher exact test. P values<0.05 were regarded as statistically significant.

Results

We obtained 101 archival breast specimens from 101 female subjects for this study. DNA from the intraductal proliferative lesions and adjacent normal breast tissue was successfully extracted in all cases. PCR was performed to amplify fragments surrounding the highly polymorphic CAG trimetric repeat in exon 1 of HUMARA, using DNA extracted from these 101 female samples as templates. Undigested and digested genome DNA from 88 of 101(87%) patients were able to be amplified successfully and 68 undigested genome DNA of 88(77.3%) were heterozygous for the HUMARA locus, as shown by the presence

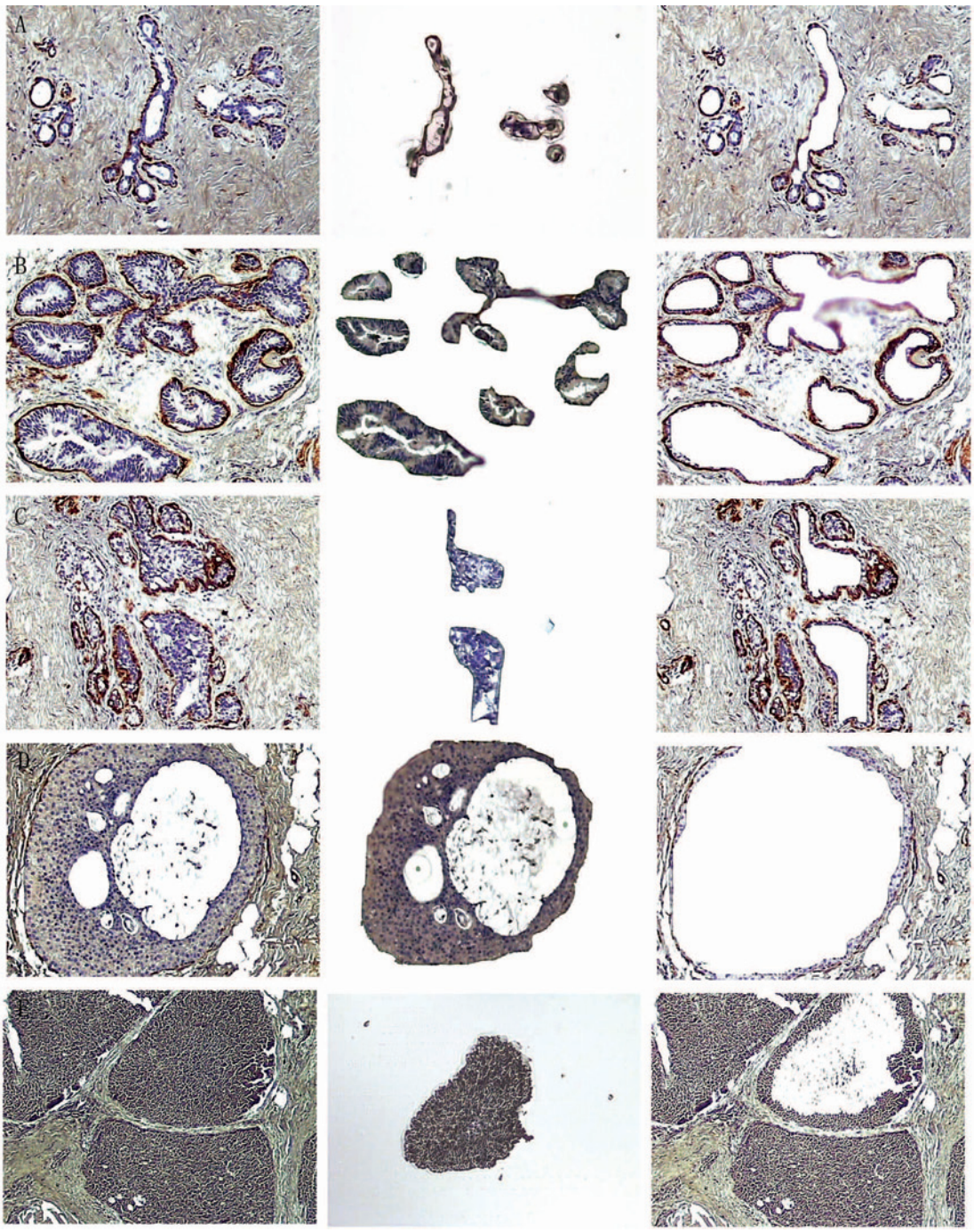

Figure 1 Tissues were microdissected using a laser Veritas™ microdissection instrument. (A) Normal breast tissue. (B) UDH. (C)DIN 1A. (D) DIN 1B. (E) Breast cancer in situ. Left column: before microdissection. Central column: the captured epithelial cells. Right column: after microdissection. (A,B,C,D:SMA immunostaining and Hematoxylin counterstaining, E: Hematoxylin staining; ×200).

of 2 major bands on electrophoretic analysis of the PCR products, and therefore, potentially informative for clonality analysis. PCR amplification of digested and undigested DNA from 9/12 UDH and 5/18 DIN 1A, yielded 2 bands of almost identical intensity (Figure 2A, B), suggesting that these tissues were polyclonal and that X-inactivation was not skewed. Digested with Hpa II blocked to amplification of one of the two HUMARA alleles in 3/12 UDH, 13/18 DIN 1A, 28/28 DIN 1B, 10/10 carcinoma in situ (Figure 2C, D), suggesting that these lesions are of monoclonal origin. DIN 1B and in situ carcinomas had

a significantly higher frequency of monoclonal origin than DIN 1A (P =0.006). Similarly, the frequency of monoclonality was significantly higher in DIN 1A than in UDH (P =0.003).

Discussion

The increasingly frequent detection of intraductal proliferative lesions by mammography has raised important questions about the pathogenetic relationship between them and breast cancer. Clonal analysis based on X-chromosome inactivation is one of the most useful methods of differentiating neoplasia from hyperplasia.

To investigate what type of intraductal proliferative lesions is already neoplasia, we have used LCM and PCR-based HUMARA assay to determine the clonal origin of these lesions. We found that 68 of 88 cases (77.3%) were heterozygous at this locus. This rate is smaller than the reported heterozygosity frequency of the HUMARA gene [13]. While Magda [20] found the heterozygous rate of his test group is 73%. This finding suggested that the polymorphism of HUMARA exon 1 is different between peoples.

There are certain limits in assessing clonality by non-random X-chromosome inactivation. On the one hand, if a sample was taken within a patch, then it shows a monoclonal pattern. Although the patch size of breast has not been determined, there is some evidence that the clonal unit is relatively small (possibly a single lobule) [21]. Our work is consistent with these data because normal breasts were found to be polyclonal. On the other hand, in rare cases, X chromosome inactivations in normal tissue occur non-randomly, responsible for a false monoclonal pattern of inactivation [14, 22]. Therefore, as a control, epithelium of normal breast gland was processed in the same manner as that of intraductal proliferative lesions [23]. Taken together, our clonality analysis can be considered reliable.

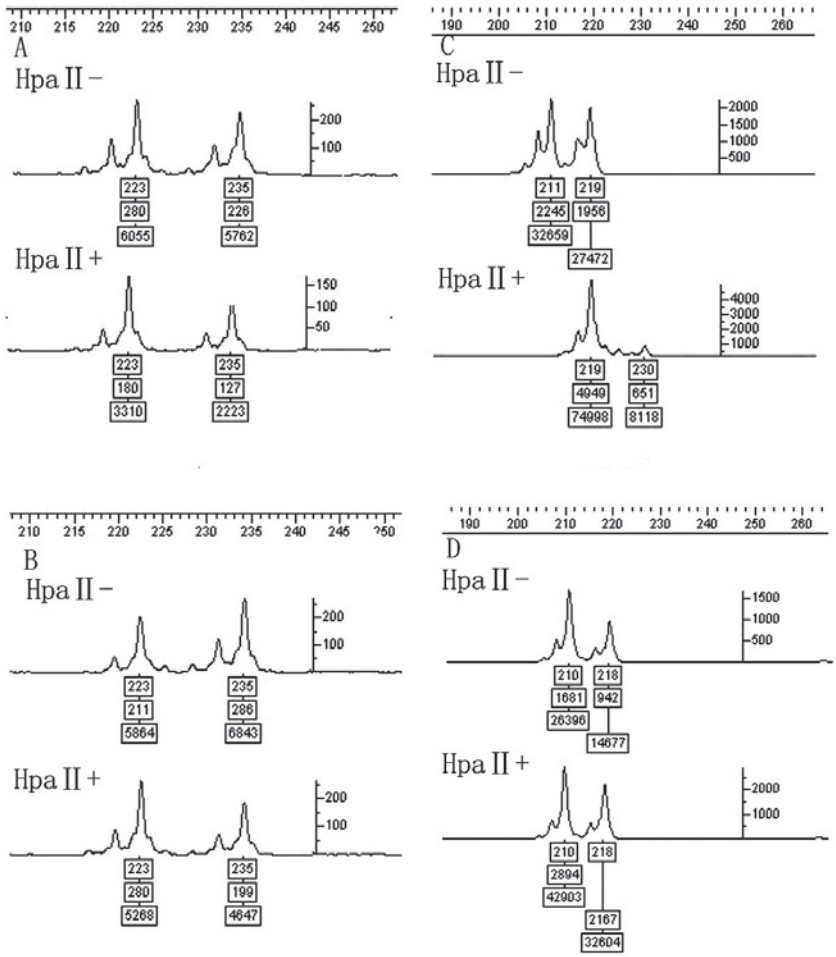

Figure 2 Representative results of clonality assays. DNA exacted from intraductal proliferative lesions and normal breast were analyzed in parallel. Epithelial cells from UDH(A) and normal breast tissue(B) exhibited the same methylation pattern with or without Hap II digestion, indicating a polyclonal origin with a CR of 0.874. Compared with normal breast tissue(D), the shorter allele is disappear after Hap II digestion in DIN 1B(D), indicating a monoclonal origin.

In the current study, 9/12(75%) UDH and 5/18(27.8%) DIN 1A showed a polyclonal X chromosome inactivation, while 3/12 UDH, 13/18 DIN 1A, 28/28 DIN 1B, 10/10 carcinoma is monoclonal. DIN 1B and in situ carcinomas had a significantly higher frequency of monoclonal origin than DIN 1A (P =0.006). Similarly, the frequency of monoclonality was significantly higher in DIN 1A than in UDH (P =0.003). These findings strongly indicated that clonal expansion is involved in an early stage of breast cancer development and DIN1 may correspond to a truly neoplasia as opposed to UDH and provide molecular support to the high risk of DIN1 for subsequent development to invasive breast cancer [24, 25]. Several data from other studies also indirectly indicated that DIN1 is one clone proliferation at the morphological or molecular level. DIN1B homogeneously expresses a CK profile of luminal type epithelial cells (CK7, CK18, CK19), while UDH displays a heterogeneous and mixed luminal (CK7, CK18,CK19) and basal phenotype (CK5 /6, CK14) [26, 27]. The vast majority of DIN1B (and low-grade DCIS) cases express high level of oestrogen receptors on nearly all cells, compared with the heterogeneous expression pattern seen in UDH.

Although the clonality analysis data indicate that a proportion of UDH lesions is monoclonal, their morphological character doesn't support it. Further, UDH hardly has genomic abnormality associated with cancer. Firstly, results obtained by means of comparative genomic hybridization reveal that breast cancers showed gross cytogenetic alterations, while UDH does not display any such changes [28, 29]. Secondly, LOH at any given locus was rare (range, 0%-15%), while in DCIS from noncancerous breasts, LOH was com-

mon, with 70% of noncomedo lesions and 79% of comedo lesions shelving at least one loss (5). Without epidemic data, it is too early to say that UDH follow a cancerous pathway.

In summary, our data allowed us to conclude that DIN 1, but not UDH, represents the obligate and direct precursor of DCIS. This would be particularly useful for better understanding of how breast carcinoma originated and even contribute to clinical management of DIN 1A, 1B.

We thank Feng Tang, Yun Bao, Zhongqing Chen, Qiong Li, and Weizhi Zhao for their help in collecting tissue specimens. We are grateful to Dr Feng Tang and Professor Hong Wang for valuable help in diagnosis.

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