Evidence of chromosomal alterations in pure usual ductal hyperplasia as a breast carcinoma precursor

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Abstract. Previous studies have shown the chromosomal alterations in usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS) in the breast with bilateral ductal hyperplasia or adjacent to invasive ductal carcinoma (IDC). However, the role of UDH as a putative precursor of breast IDC is not clear and has not been fully addressed. The aim of this study was to clarify the role of UDH in breast carcinoma pathogenesis. To investigate chromosomal imbalances and commonality, samples of pure unilateral UDH (n=20) were obtained by laser capture microdissection and analyzed by comparative genomic hybridization. Other ductal lesions, including ADH (n=2), high-grade DCIS (n=3), and grade III IDC (n=5), were assessed at the same time for comparison. The mean values of alteration were 1.95 (39/20) in UDH, 9.5 (19/2) in ADH, 11.0 (33/3) in DCIS and 18.2 (89/5) in IDC, respectively. Some common predisposition regions for the deletions were at chromosomes 1p36-pter, 13q11-14, and 16q11-23, while the high frequency amplification regions were 1q31-qter, 3p21-pter, 6p21-pter, 11q11-14, 12q11-qter, 13q21-qter, 16p12-pter, 17q12-22, and 20q. The genetic abnormalities in the spectrum of breast ductal hyperplasia revealed that the deletion of DNA copy in UDH was the lowest, and gradually increased in the lineages of ADH, DCIS and IDC. Results showed that a significant portion of UDH shares common genetic alterations with ADH, DCIS and IDC, indicating UDH as a precursor of invasive breast ductal carcinoma.

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Introduction

Clinical studies have indicated that usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) in the breast are related to different levels of risk for the subsequent development of invasive carcinoma. The risk factors of subsequent invasive breast carcinoma are 1.5 times for UDH, 4-5 times for ADH, and 8-10 times for DCIS, respectively (1,2). The conventional conception of UDH as the first precursor of invasive ductal carcinoma is based on epidemiological studies and morphological findings. Recently, a multi-step model of breast cancer pathogenesis was proposed, hypothesizing that breast cancer development occurs through a series of intermediate hyperplastic lesions from *in situ* to eventual invasive carcinoma. However, the role of UDH in the multi-step model is unclear and controversial (3,4). The World Health Organization has suggested that UDH is not a significant risk factor, and there is insufficient genetic evidence to classify it as a precursor lesion of invasive ductal carcinoma (2). No significant genetic alterations were previously detected in UDH using comparative genomic hybridization (CGH), suggesting that UDH and ADH/DCIS are not close entities (4). However, contradictory results have been reported. Major amplification of the 20q13 region occurred in all UDH cases examined in a previous study (5). Another study showed that loss of heterozygosity (LOH) exists in 37% of UDH lesions, suggesting that the development of intraductal lesions could involve a number of tumor suppressor genes (6). Moreover, it was found that there were chromosomal alterations with common losses at 1p, 16p, 17q, and 22q in ~75% of bilateral UDH (7). It was confirmed that the same copy number alterations were observed in five out of nine cases of UDH approaching ADH (8). Although UDH can be observed in bilateral breast cases, the exact incidence is unknown and most patients are unilateral. On the other hand, the frequency of LOH in breast epithelial hyperplasia was significantly higher in cases of near carcinoma than in fibroadenoma (9).

All the above indicate that the chromosomal alterations in UDH may contribute to the development of breast carcinoma.

In the present study, we investigated the chromosomal changes in 20 cases of pure unilateral UDH using comparative genomic hybridization (CGH). To reveal the relationship of genetic alterations between UDH and other ductal lesions, we also analyzed 2 cases of ADH, 3 cases of DCIS and 5 cases of IDC, for comparison. This study provides further information on the molecular genetic alterations of breast ductal lesions, and assists in understanding the molecular mechanisms for the development of breast carcinoma.

Materials and methods

Materials. Fresh tissues obtained by surgical excision from 30 patients with breast ductal lesions were collected at West China Hospital, Sichuan University, P.R. China. Samples included 20 cases of pure unilateral UDH, 2 cases of ADH, 3 cases of high-grade DCIS and 5 cases of grade III invasive ductal carcinoma, and were stored at -80˚C. The frozen sections were examined by two independent pathologists after samples were formalin-fixed and paraffin-embedded to confirm identical diagnoses. The criteria established by the International Agency for Research on Cancer (IARC) in 2003 (2), were used to diagnose the breast lesions. The clinical data regarding age, lesion location, and menopausal status of patients were collected at the same time.

Microdissection and DNA extraction. All lesions were micro-dissected from 12-μm-thick frozen sections using the laser capture microdissection system (Leica, Germany). Normal breast ductal tissue was dissected to obtain negative control DNA. Each case was extracted using the DNA Extraction Kit (Qiagen, USA). Normal control DNA was obtained from peripheral blood lymphocytes from a healthy female.

DOP-PCR and CGH analysis. Amplification of the DNA from microdissected tissue was undertaken using DOP-PCR Master (Roche, Germany). Fluorescent labeling of DNA was performed according to the CGH Nick Translation Kit procedure (Vysis, USA). The CGH probes were composed of 800 ng of test (SpectrumGreen™-labeled) and 800 ng of normal control (SpectrumRed™-labeled) DNA samples, and were co-precipitated with 40 μg of human Cot-1 DNA (Invitrogen, USA) by centrifuging at 12,000 rpm for 30 min at 4˚C to pellet the DNA. The DNA pellet was re-suspended in 3 μl purified H_2O and 7 μl of hybridization buffer (50% deionized formamide; 20% w/v dextran sulfate; 2X SSC; 0.1 mM EDTA, pH 8.0; 0.2 mM Tris-HCl, pH 7.6). The probes were denatured for 5 min at 73˚C. Normal male metaphase spreads were denatured at 73˚C for 5 min in denaturation solution (49 ml deionized formamide; 7 ml 20X SSC, pH 5.3; 14 ml purified H_2O, pH 7.0-7.5), and dehydrated through a series of alcohols. The denatured probes were hybridized to the metaphase cells under a coverslip for 72 h at 37˚C. After hybridization, the slides were washed in a solution consisting of 0.4X SSC/0.3% NP-40 (pH 7.0) at 74˚C for 5 min, placed in a solution consisting of 2X SSC/0.1% NP-40 (pH 7.0) at ambient temperature for 5 min, and then dried in darkness at ambient temperature. Lastly, the slides were stained in an anti-fade medium containing 4,6-diamino-2-phenylindole (DAPI) (Abbott Molecular Inc.). The positive control DNA (MPE 600, SpectrumGreen™-labeled, Vysis, USA) and the negative control DNA (normal breast ductal tissue, SpectrumRed™-labeled) were used for evaluating the CGH data.

Metaphase chromosome samples were observed by Leica Microsystems. Image analysis was performed using Leica CW4000 CGH software (Leica, UK). Ten representative images of high-quality hybridizations for each sample were analyzed, and an average fluorescence ratio for each chromosome was calculated. Losses and gains of genetic material were scored when the ratios were <0.80 or >1.20, respectively. The chromosomes X and Y were not analyzed.

Statistical analysis. The mean value and standard deviation were obtained for each ductal lesion group. The Student's t-test was performed to analyze differences between UDH, ADH, DCIS and IDC. Differences of p<0.05 were considered statistically significant.

Results

The complete list of chromosome alterations detected by CGH in the 30 cases is provided in Table I. A summary of CGH with respect to all 30 lesions is given in Fig. 1. The positive CGH controls of MPE600 DNA showed the losses of 1p36.1, 11q14-qter, 16q, 9p and the gain of 1q, whereas there were no detected alterations of chromosomes in the normal breast tissues. The number of DNA copy number changes of UDH ranged from 0 to 5, with a mean of 1.95±1.82 (39/20). Alterations were observed in 13 out of 20 patients. Losses were observed in chromosomes 1p, 13q, and 16q, whereas gains were observed in chromosomes 1q, 2q, 3p, 6p, 7p, 11q, 12q, 13q, 16p, 17q and 20q. In all cases of chromosomal alterations, the loss was 23.1% (9/39) and the gain was 76.9% (30/39) (Fig. 1A).

The mean of chromosomal changes in ADH was 9.50±3.53 (19/2), and the losses of DNA copy increased to 31.6% (6/19) compared with UDH (Fig. 1B). The mean of chromosomal changes was 11.0±3.60 (33/3) in high-grade DCIS, and the loss of DNA copy number in chromosomes increased to 45.5% (15/33) (Fig. 1C). In grade III IDC, the mean of chromosomal alterations was 17.8±5.50 (91/5), and the loss of DNA copy number in chromosomes reached 47.2% (42/89) (Fig. 1D).

The major common chromosomal alterations were the losses in 1p36-pter, 13q11-14, 16q11-23, and the gains in 1q31-qter, 3p21-pter, 6p21-pter, 11q11-14, 12q11-qter, 13q21-qter, 16p12-pter, 17q12-22, and 20q. In the breast lesions of ADH to IDC, some chromosomal changes were observed, such as the losses in 4q, 5q, and 17p, and the gains in 8q, 10p, 14q, and 15q.

The mean and standard deviation in UDH, ADH, DCIS and IDC were 1.95±1.82, 9.50±3.53, 11.0±3.60, and 17.8±5.50, respectively. Analyses of age, lesion location and menopausal status data compared with the CGH results in UDH were not significant (Table II). Results showed that there were significant differences between UDH vs ADH (p<0.01), UDH vs DCIS (p<0.01), and UDH vs IDC (p<0.01) (Table III).

Table I. Clinical data and chromosomal alterations of breast ductal lesions.

Case	Age	Loca	Menopause	Chromosomal alterations	Gain	Loss	Total	Mean ± SD
1	45	R	Post	1p-,3p+,7p+,17q+	3	1	4	1.95±1.82
2	43	R	Pre	None	0	0	0	
3	53	L	Post	None	0	0	0	
4	42	L	Pre	1q+,6p+	2	0	2	
5	37	L	Pre	12q+,13q+,16p-	2	1	3	
6	43	L	Pre	2q+,7p+	2	0	2	
7	56	L	Post	1q+,16q-,17q+	2	1	3	
8	67	L	Post	1p-,3p+,16p+	2	1	3	
9	34	L	Pre	11q+	1	0	1	
10	40	L	Pre	None	0	0	0	
11	46	R	Pre	None	0	0	0	
12	43	R	Pre	2q+,6p+,12q+,16q-	3	1	4	
13	60	R	Post	1p-,2q+,11q+,16q-	2	2	4	
14	49	R	Post	3p+,12q+	2	0	2	
15	27	L	Pre	20q+	1	0	1	
16	55	L	Post	None	0	0	0	
17	29	L	Pre	1q+,3p+,13q-,16p+,20q+	4	1	5	
18	42	L	Pre	None	0	0	0	
19	36	R	Pre	2q+,6p+,13q-,16p+,17q+	4	1	5	
20	48	R	Pre	None	0	0	0	
21	39	L	Pre	1q+,3p+,4q-,5q-,6p+,10p+,11q+,12q+,13q+,16p+,17q+,20q+	10	2	12	9.50±3.53
22	66	L	Post	1p-,8q+,13q-,14q+,15q+,16q-,17p-	3	4	7	
23	38	L	Pre	1p-,1q+,2q-,3q-,5q-,6q-,8q+,10p+,12q+,13q-,14q+,16q-,17p-,20q+	6	8	14	11.0±3.60
24	57	R	Post	1p-,3p+,4q-,11q-,12q-,6p+,7q+,8q+,10p+,13q+,16p+,17q+	8	4	12	
25	49	L	Post	1q+,9q-,11q+,13q-,16p+,17p-,20q+	4	3	7	
26	49	L	Post	1p-,1q+,2q+,3p-,3q+,5p-,5q+,6q-,7q+,8p-,8q+,10q-,11p+,11q-,12q+,13q+,15q+,16p+,16q-,17p-,17q+,19p-,19q+,20q-,21q+	14	12	26	17.8±5.50
27	46	L	Pre	1p-,1q+,3p-,6q-,8p-,8q+,11q+,12q-,13q +,14q-,15q-,16q-,17p-,17q+,18p-,19p+,19q-,20q+,22q-	7	12	19	
28	47	R	Post	1p-,3p+,5p+,5q+,6q-,7p+,7q+,8p-,11p+,11q-,12q+,13q-,17p-,17q+,18q+,19p-,20q+,22q-	10	8	18	
29	52	R	Post	1q+,3q+,4q-,5p+,6p+,8q+,9q+,11q-,14q-,16p+,18p-,18q+,19q+,22q-	9	5	14	
30	43	R	Pre	4q-,5p+,5q-,6p+,7p-,7q+,10p+,13q+,14q+,16q-,18q-,22q+	7	5	12	

Cases 1-20, UDH; cases 21-22, ADH; cases 23-25, DCIS; cases 26-30, grade III IDC. Loca, location of breast lesions; L, left breast; R; right breast; Pre, premenopausal status; Post, postmenopausal status; +, gain; -, loss.

Discussion

In this study, we found that there were fewer chromosomal alterations in certain cases of UDH. All UDH cases were pure unilateral lesions, and the regions of change in all aberrational cases numbered from 1 to 5 with a mean of 1.95. Our results were consistent with a previous report that the genetic alterations were the losses at 1p, 12q, 16q, 17q, 19p, 22q, and the gains at 13q in UDH (7). The mean of the chromosomal changes in this study was in agreement with the results of Gong et al (8), but the regions of chromosomal change were different. In our cases, there were no changes in chromosomes 19 and 22. This discrepancy may be due to the nature of our samples, which came from pure unilateral benign lesions. In another study, it was found that chromosomal changes in UDH included the gains on 4q, 8q, 10q, 12q, 15q, 16p, 20q and 22q, and the losses on 13q with a mean of 7.0 (5). Some of these regions were similar with our findings, but the mean was considerably higher. This could be explained by the fact that the samples in the above-mentioned study were adjacent to ductal carcinoma *in situ* and invasive ductal carcinoma. Among UDH, ADH, DCIS and IDC, the common chromosomal alterations that appeared were the losses in 1p36-pter, 13q11-14, 16q11-23, and the gains in 1q31-qter, 3p21-pter, 6p21-pter, 11q11-14, 12q11-qter, 13q21-qter, 16p12-pter, 17q12-22, and 20q. From ADH
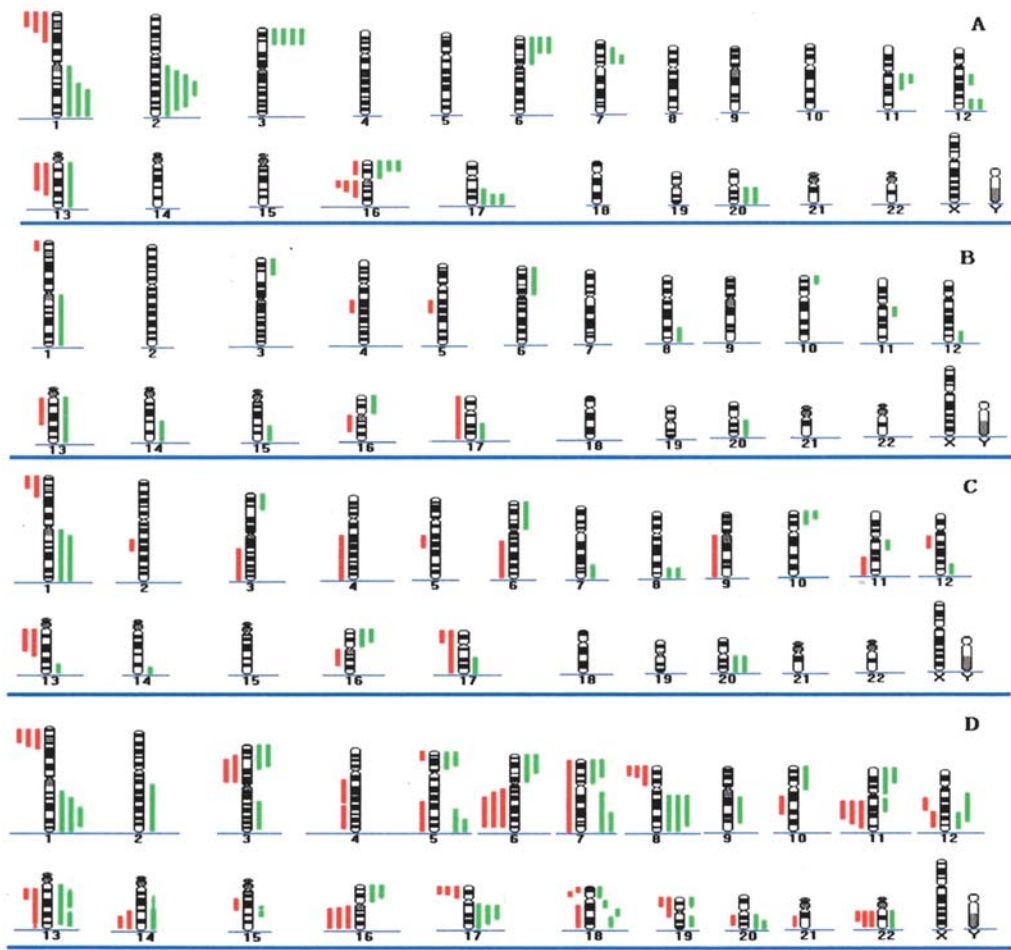
Figure 1. CGH summary of UDH, ADH, DCIS and IDC. CGH summary of (A) UDH, (B) ADH, (C) high-grade DCIS, (D), IDC. Red bars on the left of each chromosome show the regions of chromosomal losses. Green bars on the right of each chromosome indicate the regions of chromosomal gains. Ductal lesions shared some specific regions of chromosome changes. The most frequently deleted regions on the chromosomes were 1p36-pter, 13q11-14, and 16q11-23, and the most frequently amplified regions were 1q31-qter, 3p21-pter, 6p21-pter, 11q11-14,12q11-qter, 13q21-qter, 16p12-pter, 17q12-22, and 20q.

Table II. The relationship between clinical features and CGH results in UDH.

| | Total cases | Age | | Lesion location | | Menopausal status | |
	N=20	<45 (n=11)	≥45 (n=9)	L (n=11)	R (n=9)	Pre (n=13)	Post (n=7)
Total	1.95±1.82	2.09±1.83	1.78±1.76	1.67±1.60	2.38±2.00	1.77±1.92	2.29±1.70
		NS		NS		NS	
Gains	1.50±1.36	1.73±1.49	1.22±1.20	1.33±1.23	1.75±1.58	1.46±1.51	1.57±1.33
		NS		NS		NS	
Losses	0.45±0.60	0.36±0.50	0.56±0.73	0.33±0.49	0.63±0.74	0.31±0.48	0.71±0.75
		NS		NS		NS	

L, left breast; R, right breast; Pre, premenopausal status; Post, postmenopausal status; NS, not significant. All results were expressed as mean ± SD.

to IDC, some chromosomal changes emerged, such as the losses in 4q, 5q, and 17p, and the gains in 8q, 10p, 14q, and 15q. We also observed that the loss of DNA copy number was at a low level for UDH, and it increased from 23.1% to 31.6%, 45%, and 46.2%, respectively, with the progress of the ductal lesion. The frequency of LOH in UDH was inconsistent with previous findings (6).

There are few previous studies about cytogenetic changes in UDH, and the results showed some controversial data regarding the role of UDH in breast carcinoma pathogenesis (10). On the one hand, no chromosomal aberrations were observed in any of the UDH lesions, indicating that these UDH cases were benign hyperplasia (4). On the other hand, significant chromosomal aberrations have been observed in
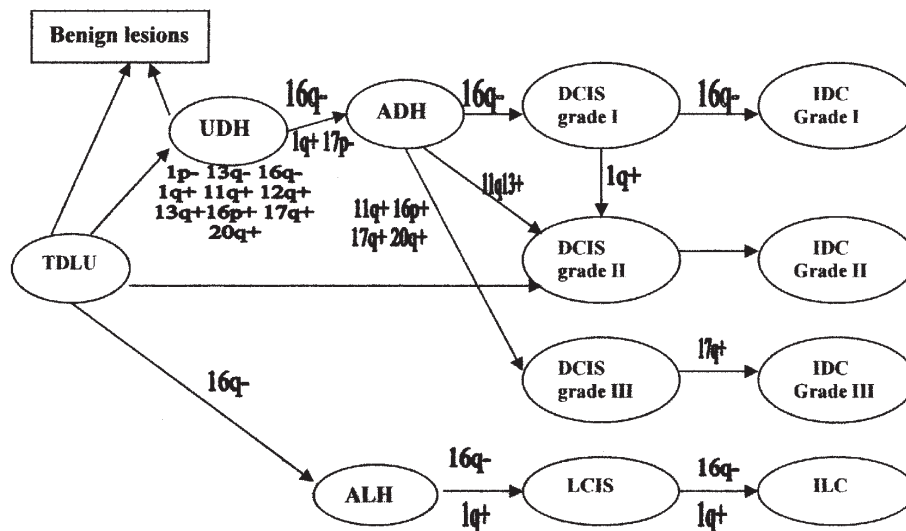
Figure 2. Hypothetical multi-step model of breast carcinoma pathogenesis showing chromosomal alterations. The main chromosomal changes were clearly different according to the lineage of breast ductal lesions.

Table III. Comparisons of UDH with ADH, DCIS and IDC.

	Mean ± SD	p value
Total		
UDH	1.95±1.82	
ADH	9.50±3.54	<0.01
DCIS	11.0±3.61	<0.01
IDC	17.8±5.40	<0.01
Gains		
UDH	1.50±1.36	
ADH	6.50±4.95	<0.01
DCIS	6.00±2.00	<0.01
IDC	9.40±2.88	<0.01
Losses		
UDH	0.45±0.60	
ADH	3.00±1.41	<0.05
DCIS	5.00±2.65	<0.01
IDC	8.40±3.51	<0.01

Analysis of UDH in relation to ADH, DCIS and IDC.

certain cases of UDH, both in our own and others, suggesting that these UDH cases are monoclonal hyperplasia (5-8). Our data indicated that at least some cases of UDH are precursors of IDC with monoclonal proliferation. The most prevalent chromosomal changes suggest that portion of UDH could serve as precursors of DCIS or IDC (5-8,35).

Furthermore, the main chromosomal changes showed some evidence of the proposed multi-step pathway of breast carcinoma pathogenesis. We found that both unilateral and bilateral (7) pure UDH shared the most common characteristics of the losses of 1p and16q, and the gain of 13q. These characteristics can also be observed in ADH, DCIS and IDC, with the only difference being in the

increasing frequencies (12,13,16). We also observed the increasing frequency of the loss of 16q and additional alterations of the loss in 17p and the gain in 1q between UDH and ADH. Comparing our data with another report (5), we observed obvious gains in 17q and 20q from ADH to high-grade DCIS as well as increasing frequency in the losses in 1p, 16q and 17p, and the gains in 1q and 13q. The main alterations were the losses in 1p, 13q, 16q and 17p, and the gains in 1q, 6p, 11q, 16p, 17q and 20q from high-grade DCIS to grade III IDC. These results showed a different pathway compared with Simpson et al (3) and Boecker et al (4) who suggested that UDH and ADH were not related to high-grade DCIS and high-grade IDC. However, according to other studies, some UDH and ADH could develop into high-grade DCIS and intermediate/high-grade IDC accompanied by alterations through the losses of 16q and 17p, and the gains in 1q, 13q, 17q and 20q (1,26,27). It has been suggested that the progress of low-grade and high-grade DCIS may undergo different molecular events. The chromosomal changes of the losses of 11q and 16q and the gain of 1q were observed exclusively in well differentiated and intermediately differentiated DCIS. Poorly differentiated DCIS displayed a higher frequency of the gains in 17q12 and 11q13. Therefore, based on the analysis of our study and previous studies (3,6-11,21), three different pathways with different molecular events in breast carcinoma pathogenesis have been hypothesized. The first pathway starts from terminal duct lobule unit (TDLU) hyperplasia, passes through UDH, ADH, and low-grade DCIS and develops into well-differentiated IDC. In this progression, the main cytogenetic changes are the losses of 16q and 11q, and the gain of 1q from low-grade DCIS turning into intermediately differentiated DCIS. The second route is clonal proliferation directly turning into intermediately differentiated DCIS with the amplification of 11q13. The third is from hyperplasia in the TDLU, through high-grade DCIS, to grade III IDC (11,28-31). The epidemiologic studies found that certain aspects of DCIS cases were associated with invasive lobular

carcinoma and certain patients diagnosed with lobular carcinoma *in situ* developed an invasive carcinoma in the long-term (32). The main genetic changes of the loss in 16q and the gain in 1p were observed in the progress of lobular hyperplasia to invasive breast cancer (30-33). Based on previous studies (3,6-11,21,28-34) and on our own findings, we hypothesized a multi-step model of breast carcinoma pathogenesis as shown in Fig. 2.

Chromosomal alterations usually lead to the gains or losses of some oncogenes and tumor suppressor genes. From the CGH data, we can screen the specific tumor suppressor genes and pro-oncogenes. In this study, with respect to the loss regions on 1p36-pter, 13q11-14, and 16q11-23, the known tumor suppressor genes are RIZ, P73, BRCA2, COL4A2, MAF, and WWOX (14,15,17-20, 22). For gain regions on 1q31-qter, 3p21-pter, 6p21-pter, 11q11-14, 12q11-qter, 13q21-qter, 16p12-pter, 17q12-22, and 20q, the known pro-oncogenes are KIF14, TRK, hTMNm, Tpr, ITGA9, ERBB2, PLCI, and STK15 (23-25,36). An understanding of these genes will provide clues for targeted therapy in breast cancer.

Acknowledgements

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