Genetic mutations and expression of p53 in non-invasive breast lesions

XIAOYUN MAO[1], CHUIFENG FAN[2], JING WEI[1], FAN YAO[1] and FENG JIN[1]

[1]Department of Surgical Oncology, Research Unit of General Surgery, Department of Breast Surgery, the First Affiliated Hospital of China Medical University; [2]Department of Pathology, the First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang 110001, P.R. China

Received June 10, 2010; Accepted August 2, 2010

DOI: 10.3892/mmr.2010.353

Abstract. Breast carcinogenesis results from the accumulation of numerous somatic genetic alterations. Although mutations of the tumor suppressor gene p53 are among the most common alterations identified in invasive breast carcinomas, it is not clear whether its alteration occurs frequently in non-invasive breast lesions, including usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS). p53 mutations were examined in 140 cases of non-invasive breast lesions, including UDH, ADH and DCIS, by high-resolution melting (HRM), followed by DNA sequence analysis. Two hundred and forty cases of non-invasive breast lesions were subjected to the immunohistochemical staining of p53 protein. The HRM and sequencing analysis demonstrated that the positive rates of p53 mutation were 0.0, 12.7 and 21.6% in UDH, ADH and DCIS, respectively. p53 protein expression was detected in none of the UDH, 14.6% of the ADH and 31.4% of the DCIS samples. Statistically, p53 mutation and protein accumulation gradually increased from UDH to ADH and to DCIS ($P<0.05$). There was a significantly positive association between p53 mutations and expression in these samples. p53 mutations and accumulation occur in non-invasive breast lesions, including ADH and DCIS, and may represent early events in breast carcinogenesis.

Introduction

The p53 tumor suppressor gene is thought to play a central role in protecting against the development of cancer. As a transcription factor, its encoding protein is a master switch that coordinates and concentrates a plethora of stress signals and transforms them into a series of responses, such as apoptosis or cell cycle arrest in response to DNA damage, thereby maintaining genetic stability in the organism (1). Mutations in p53 inactivating its function are frequently found in both familial and sporadic human cancers. More than 50% of human cancers harbor mutations in p53, and the p53 pathway is otherwise inactivated in most of the remaining cancers. In animal models, loss or mutation of p53 leads to a predisposition to a range of spontaneous and induced tumors, highlighting its protective role as a barrier to tumor development (2). Although p53 inactivation in human cancer is a complex process, depending on the tissue type, p53 dysfunction may disorder the biological events of cancer cells and give rise to their aggressive phenotypes, poor patient survival and resistance to treatment (2-6). The molecular genetic profile of p53 alterations has been well characterized in invasive breast cancer (6-8). However, only a few studies have investigated p53 alterations in non-invasive breast lesions, including usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS). Moreover, the findings of these studies differed significantly, in particular in the case of ADH.

At present, there are several long-standing methods available for the detection of mutations in oncogenes and tumor suppressor genes, including direct sequencing and single-strand conformation polymorphism (SSCP) of PCR, both of which are time-consuming procedures and require skill to interpret the results (9). High resolution melting (HRM) is a novel simple homogeneous close-tube and post-PCR method that enables genomic researchers to analyze genetic variations (SNPs, mutations and methylations) in PCR amplicons, particularly when only tiny samples are available (10-15). In HRM-designated instruments, the decrease in fluorescence caused by the transition of dsDNA to ssDNA with an increase in temperature is carefully monitored. With the aid of tailor-made analysis software, different genetic variants are discriminated by their characteristic melting curves. When melted in the presence of a saturating intercalating fluorescent dye, such as LCGreen I or Styo 9, and with the acquisition of fluorescence data over small temperature increments, amplicons containing different sequences are discriminated according to the melting transition of the PCR product and the resulting melt curve shape. Certain studies have validated the use of HRM as a method for scanning somatic mutations in p53. It is sensitive, rapid and cost effective, and markedly

Correspondence to: Professor Feng Jin, Department of Surgical Oncology, Research Unit of General Surgery, Department of Breast Surgery, the First Affiliated Hospital of China Medical University, Liaoning, Shenyang 110001, P.R. China
E-mail: jinfeng66cn@hotmail.com

Key words: non-invasive breast lesions, p53 mutation, high-resolution melting, p53 expression, immunohistochemistry

reduces the amount of sequencing required in the mutational study of p53, and thereby the cost of these studies (16-19).

Worldwide, breast cancer comprises 10.4% of all incidences of cancer among women, making it the second most common type of non-skin cancer (after lung cancer) and the fifth most common cause of cancer-related death (20). Epithelial proliferation of the breast encompasses a variety of proliferative and pre-cancerous lesions, which have to potential to progress to carcinoma. Those formed in the terminal of the ductal units are categorized as UDH, ADH and DCIS (21). To clarify genetic alterations in breast carcinogenesis, we aimed to investigate p53 mutations and its expression in a large number of non-invasive breast lesions, including UDH, ADH and DCIS samples, using HRM and immunohistochemistry.

Materials and methods

Subjects and samples. Fresh breast tissue samples were collected during surgical resection at the Department of Breast Surgery, the First Affiliated Hospital of China Medical University, China, between June 2007 and December 2009, including pure UDH (n=40), ADH (n=130) without DCIS, or DCIS (n=70) without invasive breast cancer. The samples were frozen in liquid nitrogen and then stored at -80˚C until DNA extraction. The age range was 18-82 years (mean 39.8). The study was approved by the regional ethics committee of China Medical University. Two pathologists independently reviewed each case and finally reached a unanimous diagnosis. Pathology classification was conducted according to the WHO criteria (22).

HRM assay. For each case, two H&E-stained slides of fresh frozen samples were used as guides to verify and locate the ADH and DCIS lesions. Genomic DNA was extracted from 140 cases among the 240 fresh frozen tissue samples by the phenol-chloroform method. PCR primers were designed for the flanking site of exons 5, 6, 7 and 8 of the *p53* gene (Table I) and synthesized by Sangon Ltd. (Shanghai, China). The DNA samples were further diluted to a concentration of 10 ng/μl for PCR. The PCR reaction mixture (20 μl) contained 10 ng genomic DNA, 1.5 mM $MgCl_2$ and 1X LightCycler® 480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany). Duplicate PCRs and the HRM analysis were conducted on a LightCycler® 480 PCR system (Roche Diagnostics). The PCR profile was: an initial hold at 95˚C for 15 min, followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec and extension at 72˚C for 25 sec. After the latter run, the sample was held at 95˚C for 1 min, at 40˚C for 1 min and then at 65˚C for 1 sec. This was followed by a melt step of 65-95˚C in 1˚C increments, pausing for 1 sec per step to generate the melt curve. Roche LightCycler® 480 software (ver 1.5) was used to calculate the melt peaks by plotting the negative first derivative of the primary melt curve (fluorescence vs. temperature).

Sequence analysis of HRM products. Samples with the p53 variant-scanning panels whose melting profile deviated from that of the controls (both first-pass analysis and co-amplification) were recovered from the melt profiling plate for DNA sequence analysis, purified from gel using the QIAquick gel purification kit (Qiagen), and then subjected to automated sequencing (BigDye Terminator version 3.1; Applied Biosystems) in both directions using the PCR primers. Sequences were analyzed using Mutation Surveyor 3.1 software (SoftGenetics, State College, PA, USA).

Table I. p53 primers and PCR annealing temperatures.

Exon	Sequence (5'→3')	Size (bp)
5	Fwd: TTC CTC TTC CTG CAG TAC TC Rev: CAG CTG CTC ACC ATC GCT AT	210
6	Fwd: CAC TGA TTG CTC TTA GGT CT Rev: AGT TGC AAA CCA GAC CTC AG	144
7	Fwd: GTG TTA TCT CCT AGG TTG GC Rev: CAA GTG GCT CCT GAC CTG GA	144
8	Fwd: CCT ATC CTG AGT A GT GGT AA Rev: TCC TGC TTG CTT ACC TCG CT	165

Immunohistochemistry. For immunohistochemical analysis, 4-μm-thick sections of 240 formalin-fixed, paraffin-embedded specimens were deparaffinized with xylene and rehydrated through an alcohol gradient. Immunohistochemical staining was performed with the streptavidin peroxidase system (Ultrasensitive™; Maixin-Bio, P.R. China) according to the manufacturer's instructions with a primary p53 specific antibody (DO-7, dilution 1:100; Santa Cruz Biotechnology, USA). For the negative control, sections were treated with 0.01 mol/l phosphate-buffered saline instead of the primary antibody. A slide was considered negative or positive according to the absence or presence of positive staining: no staining or <10% of total cells positive for p53 was considered negative; ≥10% of total cells positive for p53 was considered positive staining.

Statistical analysis. Differences between the positive rates were evaluated using Fisher's exact test. Analyses were performed using SPSS 13.0 statistical software for Windows. A P-value of <0.05 was considered statistically significant.

Results

A clear definition of the presence of somatic mutations in a sample was achievable by the comparison of the profile with wild-type DNA. Sequencing analysis was performed in all samples using the same PCR products after melting analysis. Figs. 1 and 2 display the results of HRM and sequencing analysis. Examples of HRM and melting profiles obtained from ADH and DCIS carried somatic mutations of exons 5-8 of p53 (Table II). We found no mutations in 40 cases of UDH, one mutation in 8 (12.7%) of 63 cases of ADH, and at least one mutation in 8 (21.6%) of 37 cases of DCIS, with one of the cases harboring two mutations simultaneously. Frequent p53 mutations were found in DCIS compared to ADH (P<0.05, Table III).

Fig. 3 displays the immunohistochemical staining of p53 protein in UDH, ADH and DCIS with the antibody against wild-type and mutant p53. p53 protein was positively expressed in the nuclei of ADH and DCIS. None of the 40 cases of UDH, 19 of 130 cases (14.6%) of ADH and 22 of 70 cases (31.4%) of DCIS showed p53 protein expression. There was a significant

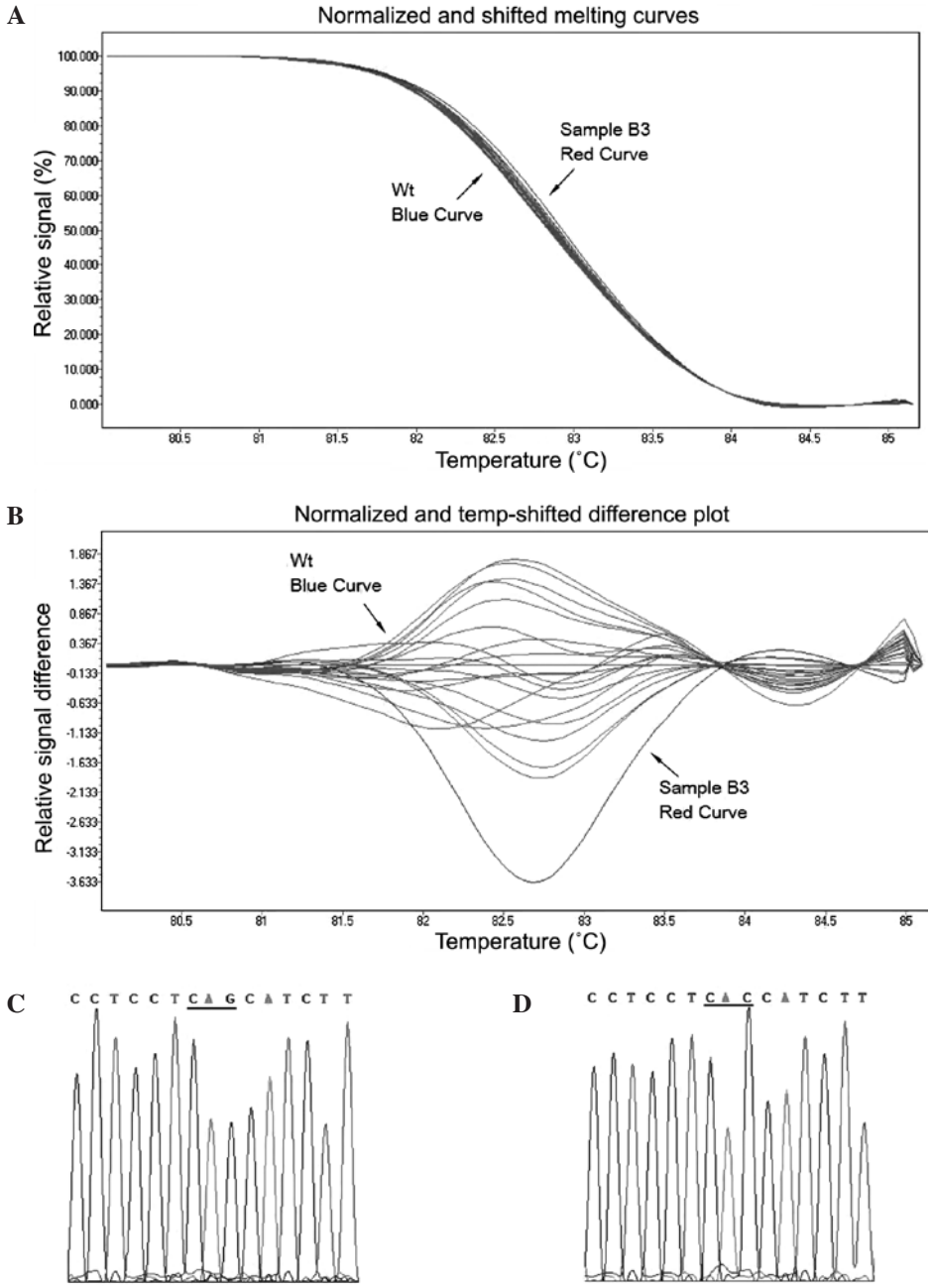

Figure 1. Screening of DNA samples of ADH for somatic mutations of p53 exon 6. (A) Normalized and temperature-shifted melting curves. (B) Substractive difference plots. (C) Sequencing trace for the wild-type of exon 6. (D) A mutation in exon 6 in sample B3 as demonstrated by sequencing analysis, CAG→CAC(G192H).

difference between the p53 protein expression of DCIS and ADH ($P<0.05$, Table III).

In 140 cases of non-invasive breast lesions, 10.7% (15/140) had both p53 expression and mutations, 84.3% (118/140) had neither mutations nor expression, 4.3% (6/140) had expression without mutations, and 1 had a p53 mutation without its expression. p53 expression was closely linked to p53 mutations (Table IV).

Discussion

Mutations of p53 during carcinogenesis may lead to an increased stability of the originally unstable p53 proteins, and p53 protein accumulation has been interpreted as a result of p53 mutation in several studies (1-5,23). Many early investigators have found a 95% rate of p53 mutation in exons 5-8, which is highly conserved through evolution, and presumably of functional importance. Although p53 mutations and protein expression have frequently been observed in invasive breast cancer and are implicated in its development, only a few studies have reported alterations of p53 in pre-invasive breast lesions (24-27). The results of these studies differed significantly, particularly in the case of ADH. Done *et al* (24,25) found that approximately 10% of DCIS carried a p53 missense mutation, and no mutations were identified in microdissected foci of epithelial hyperplasia, including one sample with atypia, suggesting that p53 mutations usually occur before the invasion of breast carcinomas. It should be noted that the authors only examined one sample of epithelial hyperplasia with atypia in their study. Keohavong *et al* (26)

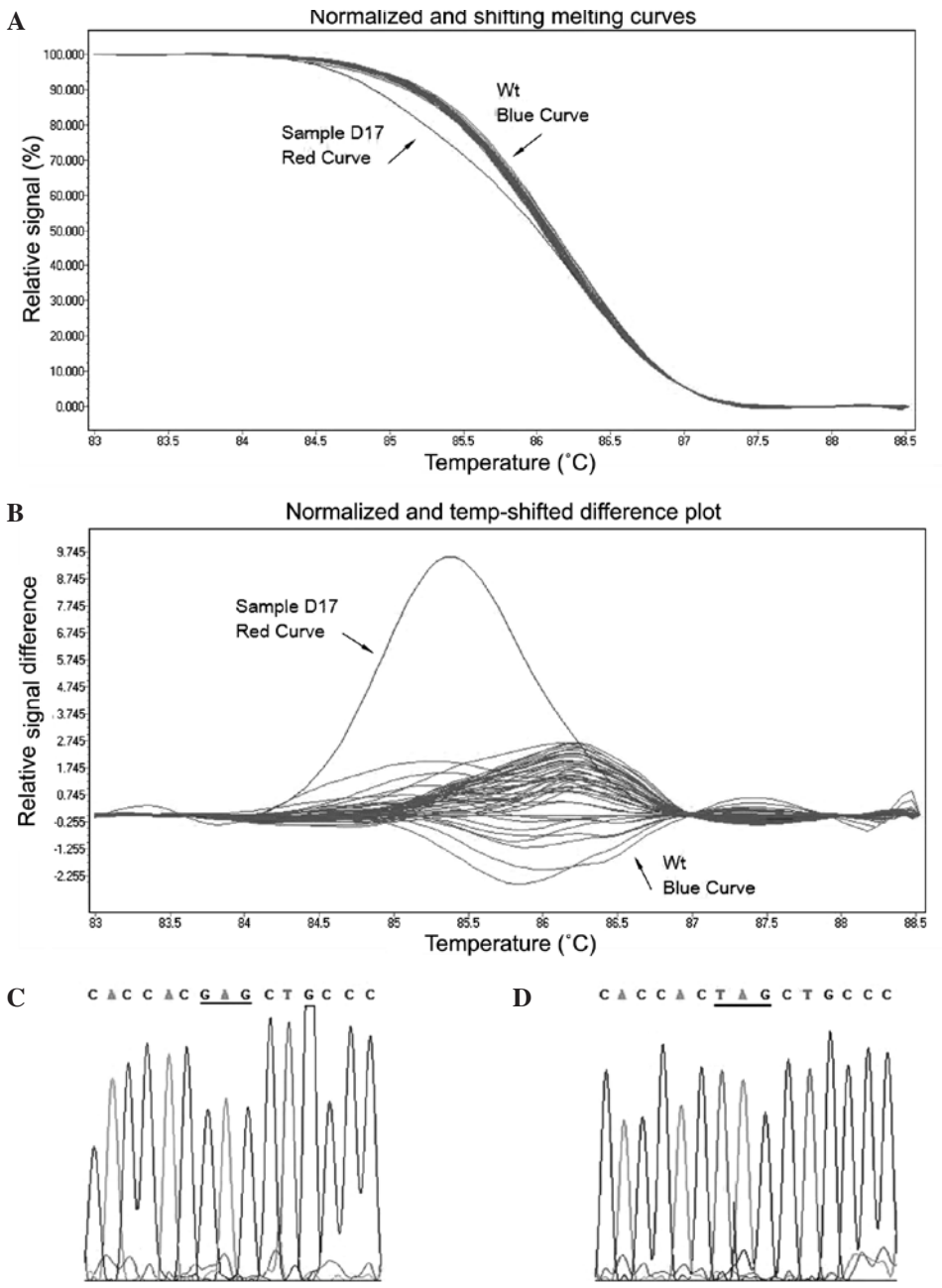

Figure 2. Screening of DNA samples of DCIS for somatic mutations of p53 exon 8. (A) Normalized and temperature-shifted melting curves. (B) Substractive difference plots. (C) Sequencing trace for the wild-type of exon 8. (D) A mutation in exon 8 in sample D17 was demonstrated by sequencing analysis, GAG→TAG(G298END).

combined tissue microdissection and SSCP to investigate p53 mutations in paraffin-embedded breast tissue sections, including 6 cases of DCIS and 10 of ADH. p53 mutations were detected in 3 cases of DCIS and in 5 cases of ADH, indicating that p53 is genetically altered not only in DCIS, but also in ADH. However, data remain scarce, and it is necessary to further investigate p53 mutations and expression in non-invasive breast ADH and DCIS using a large sample, including a number of non-invasive breast lesions.

HRM has high sensitivity and specificity, with a performance as good as, if not better than, other commonly used mutation scanning techniques (28). It is a closed tube system that not only reduces the potential for contamination, but also increases sample throughput, as it is not necessary to physically separate the DNA molecules. HRM furthermore offers significant savings in cost and turnaround time, and is an attractive choice for p53 mutation scanning, especially in high-throughput environments. Sonia *et al* (16) undertook mutation screening of the entire p53 locus by HRM and direct sequencing using a set of 47 samples, and found the sensitivity and specificity of HRM for sequence variant detection to be 1.0 and 0.83. These results suggest that HRM provides sensitive assays for the detection of new sequence variants and the genotyping of known polymorphisms.

Here, we examined mutations of p53 exons 5-8 by HRM, and found its mutations in 16 samples. Sequence analysis indicated that 1 case was a nonsense mutation and 15 cases were missense mutations. p53 gene mutations occurred in 12.7% of the ADH samples and 21.6% of DCIS, but did not occur in UDH.

Table II. p53 mutation and expression analysis of ADH and DCIS.

Simple ID	Types	HRM and sequencing results				p53 protein expression
		HRM	Exon mutation	Predicted effect	No. of cited db	
B3	ADH	MUT	6, CAG-CAC	G192H	5	+
B8	ADH	MUT	6, CCC-CTC	P219L	7	+
A1	ADH	MUT	8, ACA-CCA	T284P	12	+
A13	ADH	MUT	5, CGC-CAC	R175H	1,262	+
A22	ADH	MUT	7, GGC-GAC	G245D	188	+
A23	ADH	MUT	7, ATG-ATA	M237I	126	+
C1	ADH	MUT	8, GTT-GCT	V274A	21	+
C5	ADH	MUT	6, CAT-CCT	H193P	19	+
C6	ADH	MUT	6, no mutation	No	No	-
D1	DCIS	MUT	7, TCC-TGC	S241C	37	+
D7	DCIS	MUT	8, CGT-TGT	A273C	744	+
D9	DCIS	MUT	8, GAC-AAC	D281N	39	+
D10	DCIS	MUT	6, CTT-CGT	L194R	69	+
			6, GTG-ATG	V216M	80	
D11	DCIS	MUT	5, CCC-CAC	P151H	62	+
D17	DCIS	MUT	8, GAG-TAG	G298END	76	-
D21	DCIS	MUT	7, CGG-CAG	R248Q	952	+
D25	DCIS	MUT	5, CAT-CGT	H179R	161	+

No. of cited db, number of times the mutation has been found in human cancers in the p53 database.

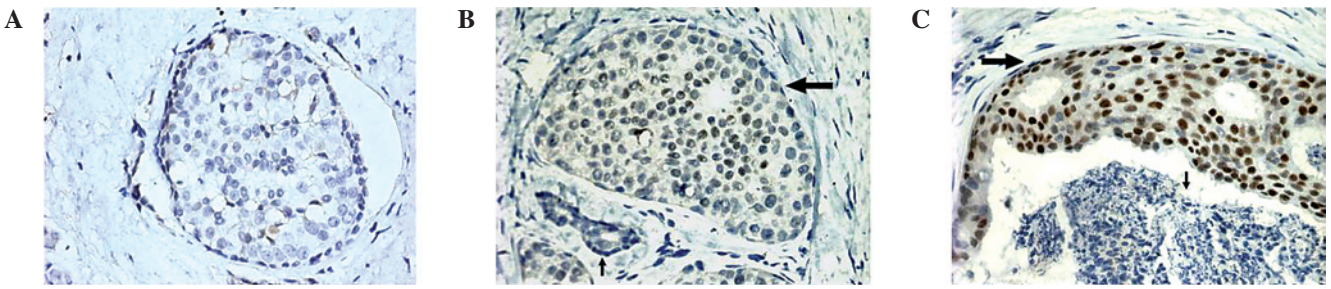

Figure 3. Immunohistochemical staining of p53 protein in non-invasive breast lesions. (A) p53-negative staining in UDH. (B) p53-positive staining in ADH. The big arrow indicates a breast duct filled with cells with atypical hyperplasia. The cells are fairly identical in size and shape. p53 positivity was observed in some nuclei (>10%). The small arrow indicates a normal duct without p53 expression. (C) p53-positive staining in DCIS. The big arrow indicates a DCIS with positive staining of p53 in the nuclei (>10%). The small arrow indicates necrosis in the DCIS.

Table III. Mutation and expression of p53 in breast carcinogenesis.

Groups	p53 mutation		p53 expression	
	Total no.	No. (%)	Total no.	No. (%)
UDH	40	0 (0.0)	40	0 (0.0)
ADH	63	8 (12.7)	130	19 (14.6)
DCIS	37	8 (21.6)[a]	70	22 (31.4)[a]

[a]P<0.01, compared to ADH.

Immunohistochemically, p53 protein was not expressed in UDH, but was observed in 14.6% of ADH and 31.4% of DCIS samples. Cases with a nonsense p53 mutation had no p53 expression. p53 expression was closely linked to p53 mutations in non-invasive breast lesions, in agreement with the interpretation of mutant p53 protein accumulation as a result

Table IV. Comparison of p53 expression with its mutations in breast lesions.

p53 mutation	p53 expression		Total
	+	-	
+	15	1	16
-	6	128	124
Total	21	119	140

P<0.01.

of its mutation. Additionally, there was a gradual increase in the incidence of p53 mutations and its expression from UDH to ADH and to DCIS, supporting the hypothesis that p53 mutations and subsequent accumulation may be involved in the tumorigenesis of breast tissue as an early event in ADH. The low frequency of p53 mutations in ADH compared to DCIS also supports the epidemiological evidence that these lesions have distinct differences, and may in part explain the different relative risks of invasive carcinoma.

In summary, p53 mutations and consequent protein accumulation may be involved in breast tumorigenesis. It may be useful to monitor genetic alterations and aberrant p53 expression in pre-cancerous lesions of the breast.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 30950009). J.F. and M.X.Y. designed the research and wrote the paper. M.X.Y. and F.C.F. collected the breast lesion tissues and carried out the experiments. W.J. and Y.F. analyzed the data.

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