Increased expression of centrosomal α, γ-tubulin in atypical ductal hyperplasia and carcinoma of the breast

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Centrosomal abnormalities have been found in various cancer types. We sought to determine whether centrosomal dysfunctions occur in the atypical ductal hyperplasia (ADH)–carcinoma sequence of breast cancer. As α and γ-tubulins are the structural components of centrosomes, we performed real time quantitative polymerase chain reaction (qPCR), *in situ* hybridization (ISH) and immunnohistochemistry (IHC) to determine the DNA copy levels, messenger RNA (mRNA) expression, and protein expression of α and γ-tubulins respectively. γ-tubulin staining was used for the localization and quantification of centrosomes. We found that *α-tubulin* or *γ-tubulin* mRNA was increasingly expressed from normal breast tissue (NBT) to ADH, ductal carcinoma *in situ* (DCIS), and infiltrative ductal carcinoma (IDC), respectively, with the highest expressions being found in DCIS. The expression profiles of α, γ-tubulin proteins were concordant with that of mRNA, except that the highest expression was found in IDC. Similarly, DNA copies of α, *γ-tubulins* showed a rising tendency, with the highest level for γ-tubulin attained in IDC and that for α-tubulin was found in DCIS. However, there was no significant difference of α, γ-tubulin DNA copy levels, mRNA expression, and protein expression between DCIS and IDC. Our results demonstrate that centrosomal aberrations may play key roles in the early stage of breast tumorogenesis. The malignant transformation sequence is probably attributable to the amplification of centrosomal DNA leading to mRNA and protein over-expression of these centrosomal proteins. Furthermore, determination of α, *γ-tubulins* using combined qPCR with ISH may be useful in assisting the diagnosis of premalignant lesions of the breast. (Cancer Sci 2009; 100: 580–587)

Breast cancer is the leading cancer among women in Europe and America. In China, epidemiologic studies showed that the incidence of breast cancer has been increasing over the last 20 years, and the standardized rate of the annual incidence has greatly increased from 17 to 24 per 100 000 from 1980 to 2000,[1] with a higher increasing rate for young patients. The role of centrosomes in a wide range of tumor types has been investigated[2,3] and centrosome aberration (supernumerary and structurally altered centrosomes) may play a potentially causative role in malignant progression. Centrosomes are involved in diverse cellular activities, especially in the assembly of the bipolar mitotic spindle and maintaining the genomic fidelity of the daughter cells.[4] It has been hypothesized that dysfunction of centrosomes may lead to chromosomal instability[5,6] and other cellular changes that may be important in the development of malignant tumors.[7] Some researchers found that centrosome defects were present in a significant fraction of precursor lesions, including *in situ* carcinomas of the uterine cervix, prostate, and female breast by detecting centrosome proteins, pericentrin and γ-tubulin using immunohistochemistry.[8–10] Moreover, supernumerary centrioles (more than four centrioles), structurally altered centrosomes, changes of microtubule nucleating capacity, and aberrant phosphorylation of centrosome protein[11] were found in breast tumor compared to corresponding normal tissue.

We have previously investigated the potential mechanism for the transition of pre-invasive lesions to early carcinoma in atypical ductal hyperplasia (ADH) and peripheral papilloma with atypical hyperplasia (PPAH).[12–14] We showed by immunofluorescence that there was a distinctive increase in the expression of α and γ-tubulins with the transition of normal epithelium to ADH to ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC).[15] In the current study, we analyzed the role of centrosomal aberrations in breast carcinogenesis. We studied a large series of normal breast tissue (NBT), ADH, DCIS and IDC samples of breast by assessing the expression level of α, γ-tubulin proteins and messenger RNA (mRNA), as well as the DNA copy numbers of these two genes quantitatively in these samples.

Materials and Methods

Tissue specimens. Tissue collection and analysis in this study were approved by the Ethical Committee of Tianjin Medical University Tumor Hospital, China. All cases of breast surgical specimens, formalin-fixed and paraffin-embedded, were anonymized after collection from the archival file of the Breast Pathology Department, Tianjin Tumor Hospital. Eighty cases of ADH/PPAH, 80 of DCIS and 80 of IDC were randomly selected from January 1998 to June 2006. Sixty cases of NBT were obtained from the cases that underwent wide local excision for fibroadenomas. The pathologic diagnosis had been confirmed by two senior pathologists according to the 2003 WHO histological classification of tumors of the breast.[16]

In situ hybridization (ISH) was performed in 40 cases of ADH/PPAH, 40 of DCIS, 40 of IDC and 30 of NBT that were randomly selected from the above groups. Real time quantitative polymerase chain reaction (qPCR) was performed in 20 cases of ADH/PPAH, 20 of DCIS, 20 of IDC and 20 of NBT that were randomly chosen from the ISH groups.

Detection methods and standardization assessment.

In situ hybridization. To detect the expression of *α-tubulin* and *γ-tubulin* mRNA, specific dignoxigenin (DIG)-labeled oligonucleotide probes were generated. The sequences of *γ-tubulin* and *α-tubulin* probes were 5′CCAAC TGTAG GGTGA TGAAT

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TCTCT CGGC and GAGGG TGAGG AAGAA GGAGA GGAAT ACTA respectively. ISH analysis was preformed according to the manufacturer's instruction with minor modification. Briefly, 4 μm paraffin sections were dewaxed and rehydrated, dipped in 0.2 M hydrochloric acid for 10 min, pretreated with proteinase K compound digesting solution for 5 min at 37°C. Then the sections were incubated at 37°C for 2 h with pre-hybridization working solution provided by the *in situ* hybridization kits (HanYang Biotech Co.), followed by pipetting the hybridization solution containing *α-tubulin/γ-tubulin* probes on the sections. Hybridization was performed in a humidity chamber at 37°C and incubated overnight. After hybridization, the sections were rinsed in 2 × SSC for 5 min, 3 times, 0.2 × SSC for 5 min, 3 times, 0.1 M Tris Buffered Saline (TBS) for 5 min, 3–5 times. Thereafter the sections were incubated with biotinylated anti-Digoxin (DIG) antibody, and subsequently high sensitivity avidin-biotin peroxidase complex at 37°C for 30 min. The hybridization signal was developed in the dark with 3.3'-diaminobenzidine (DAB). Positive controls (tumor known to be positive for *α/γ-tubulin* expression) and negative controls (using known positivity sample digested with RNase or using hybridization solution without a probe) were included in every run.

The sections were observed by light microscopy. Five high-power fields (× 400) were chosen per case and 150–200 cells were counted for each power field. The scoring system was modified and used according to evaluation standards.[17] The percentage of the staining cells (P) was scored as follows: 0 (staining of <5% of cells), 1 (5–25% of cells), 2 (25–50%), 3 (50–75%), and 4 (75–100%). Staining intensity (I) was graded as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (intense staining). Samples in each power field were evaluated for both factors, that is, P multiplied by I. The scoring of each case was a mean value of that of five power fields. Eventually the sections were graded as follows: (–) scoring 0–2, 1 + scoring 2–5, 2 + scoring 5–8, 3 + scoring over 8.

Immunohistochemistry. Tissue sections (5 μm) were deparaffinized and rehydrated using standard procedures. Subsequently, tissue sections were incubated with a primary monoclonal antibody (anti-α-tubulin antibody at 1:100, anti-γ-tubulin antibody at 1:500, Santa Cruz Laboratories), and followed by incubation with a biotinylated secondary antibody and streptavidin-HRP (horseradish peroxidase) conjugate tertiary antibody for 15 min at 37°C. The sections were then treated with freshly prepared DAB to visualize the signal. Positive controls and negative controls were included in every run of the experiment.

γ-tubulin was used for centrosome localization and quantification because it decorates spindle poles, whereas a large fraction of α-tubulin was cytoplasmic and obscured the spindle microtubule signal. The evaluation of centrosomes was based on the criteria established by Lih-Ching Hsu[18] and Stephen J. Doxsey.[19] A tumor was considered abnormal when centrosome abnormalities were observed in at least eight microscopic fields (1000×) within one sample.

Quantitative real time PCR of genomic DNA. (1) Primer designs. Specific primers were designed according to the published sequence of α-tubulin and *γ-tubulin* gene in the National Center for Biotechnology Information (NCBI) database using the primer design software program Oligo 6.72. BLAST analysis was performed to exclude the influence of pseudogenes. *α-tubulin* was amplified using the forward (5′-TAT CGA GCG CCC AAC CTA CAC T-3′) and reverse (5′-CAC CAG GTT GGT CTG GAA TTC TGT C-3′) primers, which generate a fragment of 127 bp in length. Similarly, *γ-tubulin* was amplified using the forward (5′-GCG ATG CCG AGG GAA ATC ATC ACC CT-3′) and reverse (5′-TGG GTG CCC CAG GAG ATG TAG TCT G-3′) primers, which generate a fragment of 135 bp in length. The external reference gene (*α-tubulin*) was amplified with the following primers: 5′-TAT CGA GCG CCC AAC CTA CAC T-3′ and 5′-CCT CAC CCT CTC CTT CAA CAG AAT C-3′ with generated fragments of 683 bp in length.

(2) DNA extraction. Breast tissue samples were prepared by conducting microdissection of tissue sections. Genomic DNA was extracted using a commercial DNA extraction kit (E.Z.N.A. Tissue DNA Kit, Omega Bio-tek) according to the manufacturer's instructions. The quality and concentration of extracted DNA was estimated from absorbance at 260 nm and 280 nm measured on the spectrophotometer. Isolated DNA was kept at –20°C.

(3) qPCR assay. PCR amplification was carried out in a final volume of 25 μL containing 2× Premix Taq 12.5 μL, 1 μL of each primer and genomic DNA 100 ng. The PCR reaction conditions were as follows: DNA was denatured at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min before stopping at 4°C. The amplified fragments were detected by 1% (w/v) agarose gel electrophoresis followed by ethidium bromide staining. Each band was analyzed using Kodak 2D image-analysis software. Recombinant plasmid containing each amplified product was used to generate a standard curve.

A GeneAmp 5700 Sequence Detection System was used to perform qPCR amplification; PCR amplifications were carried out in a final volume of 20 μL containing 10 μL SYBR Green premix (Takara Co.), 0.8 μL of each primer and 80 ng of genomic DNA. Amplification condition was 94°C for 5 min then 40 cycles at 94°C for 30 s, 57°C for 20 s, and 72°C for 20 s. Negative controls were performed by omitting templates or primers. Real-time PCR reactions were done in triplicate for each sample, and absolute DNA quantification was calculated using the standard curve method. Reactions were carried out with different concentrations of one standard plasmid. DNA copy numbers of the samples to be tested were deduced by comparing the Cycle Threshold (CT) value of the samples to that of a known concentration of standard plasmid. Data analysis was performed using Microsoft Excel software.

Statistical analysis. The data analysis was performed with the SPSS11.5 software package. Ridit Analysis, Wilcoxon rank-sum test and χ^2 test were used to compare the results of ISH and IHC. DNA copy numbers were given logarithmic transformation to arrive at normal distribution. One-way analysis of variance (ANOVA) was used to compare differences among four groups. Least significant difference *t*-test was used for multiple comparisons. The level of significance was set at 0.05.

Result

ISH for *α-tubulin* and *γ-tubulin*. The expression levels of *α-tubulin* or *γ-tubulins* mRNA in NBT, ADH/PPAH, DCIS and IDC were detected by *in situ* hybridization and are presented in Fig. 1(a–d), Fig. 2(a–d) and Table 1. There is a distinctive increase in the expression levels of α, *γ-tubulins* mRNA in ADH/PPAH when compared to NBT. The expression levels of these two genes in DCIS and IDC were similar, and was significantly ($q = 7.7145$, $P < 0.05$ for *α-tubulin* and $q = 7.3562$, $P < 0.05$ for *γ-tubulin*) higher when compared to ADH/PPAH. Table 1 shows that the highest expression rate was found in DCIS (82.5% for α-tubulin and 85.0% for *γ-tubulin*). IDC shows a slightly lower expression of the two genes (77.5% for *α-tubulin* and 82.5% for *γ-tubulin*). The positive score and staining intensity of the expressions showed significant differences between any two consecutive groups, except between IDC and DCIS (Table 2).

The mRNA expression of *α-tubulin* did not differ from that of *γ-tubulin* in NBT, ADH/PPAH, DCIS and IDC ($\chi^2 = 0.0770$, 0.2083, 0.0918, 0.3125, respectively, ($P > 0.05$) (Fig. 3).

IHC for α-tubulin and γ-tubulin. The expression levels of α-tubulin and γ-tubulin as determined fby immunohistochemistry are shown in Table 3, Fig. 4(a–d) and Fig. 5(a–d). Significant differences in the expression of α-tubulin and γ-tubulin were

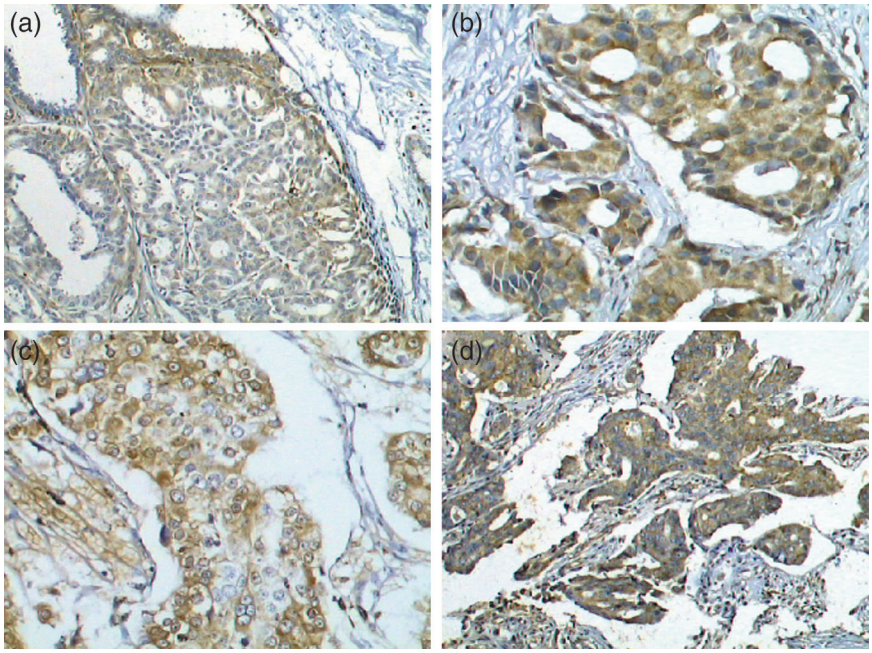

Fig. 1. The expression of *α-tubulin* messenger RNA (mRNA) in various lesions of the breast. (a) Positive expression (+) of *α-tubulin* mRNA observed as brown-yellow color in the cytoplasm in atypical ductal hyperplasia (ADH)/peripheral papilloma with atypical hyperplasia (PPAH) (*in situ* hybridization [ISH]); (b) Positive expression (+ + +) of *α-tubulin* mRNA observed as brown-yellow color in cell cytoplasm in ADH/PPAH (ISH); (c) Positive expression (+ +) of *α-tubulin* mRNA observed as brown-yellow color in the cytoplasm in ductal carcinoma *in situ* (ISH); (d) Positive expression (+ + +) of *α-tubulin* mRNA observed as brown-yellow color in the cytoplasm in invasive ductal carcinoma (ISH).

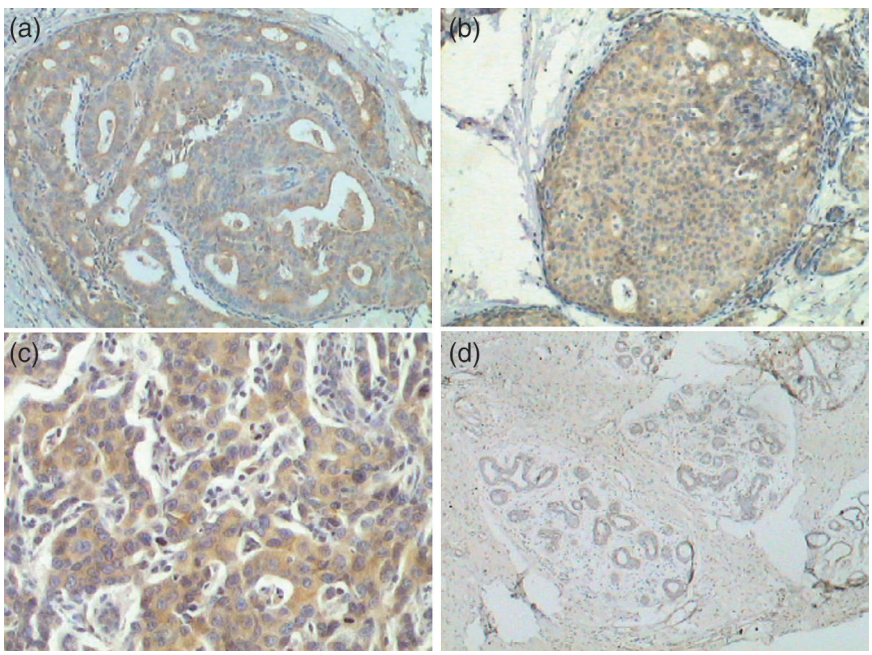

Fig. 2. The expression of γ-tubulin messenger RNA (mRNA) in the various lesions of the breast. (a) The positive expression (+ +) of γ-tubulin mRNA observed as brown-yellow color in the cytoplasm in atypical ductal hyperplasia/peripheral papilloma with atypical hyperplasia (*in situ* hybridization [ISH]); (b) The positive expression (+ + +) of γ-tubulin mRNA observed as brown-yellow color in the cytoplasm in ductal carcinoma *in situ* (DCIS) (ISH); (c) The positive expression (+ + +) of *γ-tubulin* mRNA observed as brown-yellow color in the cytoplasm in invasive ductal carcinoma (ISH); (d) The expression (±) of *γ-tubulin* mRNA observed in the cytoplasm in normal breast tissue (ISH).

Table 1. Messenger RNA expression of *α-tubulin* and *γ-tubulin*

Groups in the histology	No.	α-tubulin					γ-tubulin				
		–	+	+ +	+ + +	% positive	–	+	+ +	+ + +	% positive
NBT	30	20	9	1	0	33.3	21	8	1	0	30.0
ADH/PPAH	40	15	15	7	3	62.5	17	14	7	2	57.5
DCIS	40	7	8	18	7	82.5	6	14	13	7	85.0
IDC	40	9	13	12	6	77.5	7	9	15	9	82.5
Total	150	51	45	38	16	66.0	51	45	36	18	66.0

Abbreviations: NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

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detected between NBT and ADH/PPAH groups ($P = 0.000$). Similar levels of expression were detected in DCIS and IDC, and the expression levels were significantly higher than ADH/PPAH and NBT. The expression levels of α-tubulin and γ-tubulin showed positive correlation (r = 0.866, $P = 0.000$).

Centrosome presentation in NBT and ADH-carcinoma sequence of breast are shown in Fig. 6(a–d). The results of centrosome abnormality analyses are summarized in Table 4. Centrosome structure abnormalities included that there were more than two centrosomes in >5% of the cells examined, they had a diameter greater than twice the diameter of centrosomes present in normal epithelium within the same section, the ratio of their greatest and smallest diameter exceeded 2 or they were abnormal in shape, for example elongated. Centrosome function was considered abnormal if there were abnormalities of centrosome structure. Significant differences were found among NBT, ADH, DCIS and IDC ($\chi^2 = 78.067$, $P = 0.000$). Centrosome abnormality and the expression levels of γ-tubulin protein showed positive correlation (r = 0.848, $P = 0.000$).

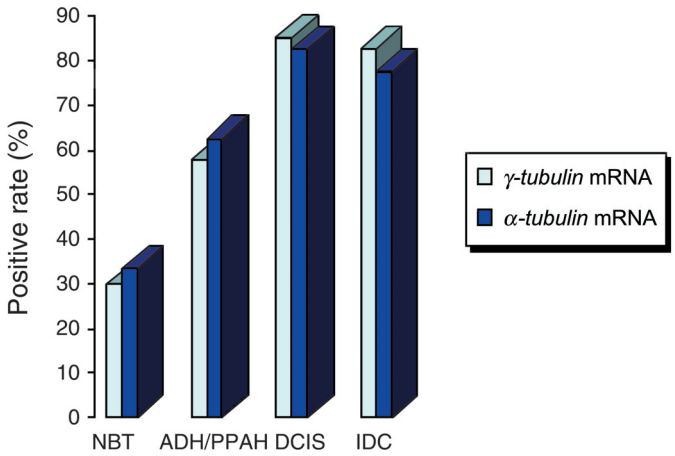

Fig. 3. Comparison of positive rate of *α-tubulin* and *γ-tubulin* messenger RNA expression in each group. There were no significant differences between *α-tubulin* and *γ-tubulin* in any group. NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

Table 2. Multiple comparison of α-, *γ-tubulin* messenger RNA expression in four groups

Ri-Rj	α-tubulin		γ-tubulin	
	χ^2	*P*	χ^2	*P*
NBT – ADH/PPAH	5.8333	0.017	5.2241	0.020
NBT – DCIS	17.4900	0.000	21.8863	0.000
NBT – IDC	13.7812	0.000	19.6875	0.000
ADH/PPAH – DCIS	4.0125	0.040	7.3837	0.005
DCIS – IDC	0.0039	>0.05	0.0918	>0.05
ADH/PPAH – IDC	8.1481	0.000	5.9524	0.015

Abbreviations: NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

ISH versus IHC. Comparing the mRNA and protein expression of α-tubulin, no significant differences were observed between the NBT, ADH/PPAH and DCIS groups (z = 0.1127, 0.0302, 0.0674, $P > 0.05$) (Fig. 7). In IDC, α-tubulin mRNA expression was lower than protein expression, in which the staining intensity showed a statistically significant difference (z = 4.0246, $P < 0.05$). Nevertheless, there was no significant difference in the positive rate between mRNA expression and protein expression of α-tubulin in IDC ($\chi^2 = 2.0289$, $P = 0.180$).

Comparing the results of ISH and IHC for γ-tubulin, staining intensity and positive rate of mRNA in NBT, ADH/PPAH, DCIS and IDC showed no significant difference from that of the protein level (z = 0.1437, 0.0177, 0.0822, 1.2591, $P > 0.05$) (Fig. 8). Although the positive rate of mRNA was lower than that of protein in IDC, it showed no statistically significant difference ($\chi^2 = 0.0981$, $P = 0.780$).

qPCR of DNA copy number for *α-tubulin* and *γ-tubulin*. Quantitative PCR was performed to evaluate the DNA copy number of α-tubulin and γ-tubulin. The PCR products were resolved by agarose gel electrophoresis and only one bright band of expected size was observed (Figs 9 and 10). Melting curve analysis also showed a single peak curve, suggesting the amplification is specific. The amplification curves and standard curves of qPCR of an external reference gene are presented in Figs 11 and 12.

The mean values of DNA copy numbers in the four groups are shown in Table 5. DNA copy numbers increased 1.1 and 1.24 times from NBT to ADH/PPAH, 1.7 and 2.24 times from NBT to DCIS, and 0.29 and 0.49 times from ADH/PPAH to DCIS for α-tubulin and *γ-tubulin* respectively, and this increase was significant (F = 25.543 for *α-tubulin* and 93.3102 for *γ-tubulin*, $P = 0.000$). There was no significant difference in DNA copy numbers of α, *γ-tubulins* between DCIS and IDC (Fig. 13).

Discussion

In his famous book, *The Origin of Malignant Tumors*, published in 1914, Boveri hypothesized that multipolar mitoses cause aneuploidy. Events resulting in supernumerary centrosomes can lead to multipolar spindles and lead to improper segregation of the sister chromatids and generate aneuploid daughter cells.[20] It was not until Fukasawa *et al.*[21] observed that centrosomal dysfunction was a downstream consequence of p53 nullizygosity in mouse Mouse Embryonic Fibroblast (MEF) cells, and the subsequent verification in animal models and tumor specimens some 80 years later that centrosomes became the focus in the research of tumor cell biology and molecular pathology, with their dysfunction being found in diverse types of tumor and tumor cell lines.[22]

Table 3. Protein expression of α-tubulin and γ-tubulin

Groups in the histology	No.	α-tubulin					γ-tubulin				
		–	+	+ +	+ + +	% positive	–	+	+ +	+ + +	% positive
NBT	60	41	18	1	0	31.7	39	19	2	0	35.0
ADH/PPAH	80	28	25	23	4	65.0	33	27	15	5	58.8
DCIS	80	11	16	28	25	86.3	11	22	28	19	86.3
IDC	80	10	12	32	26	87.5	12	12	35	21	85.0
Total	300	90	71	84	55	70.0	95	80	80	45	68.3

Abbreviations: NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

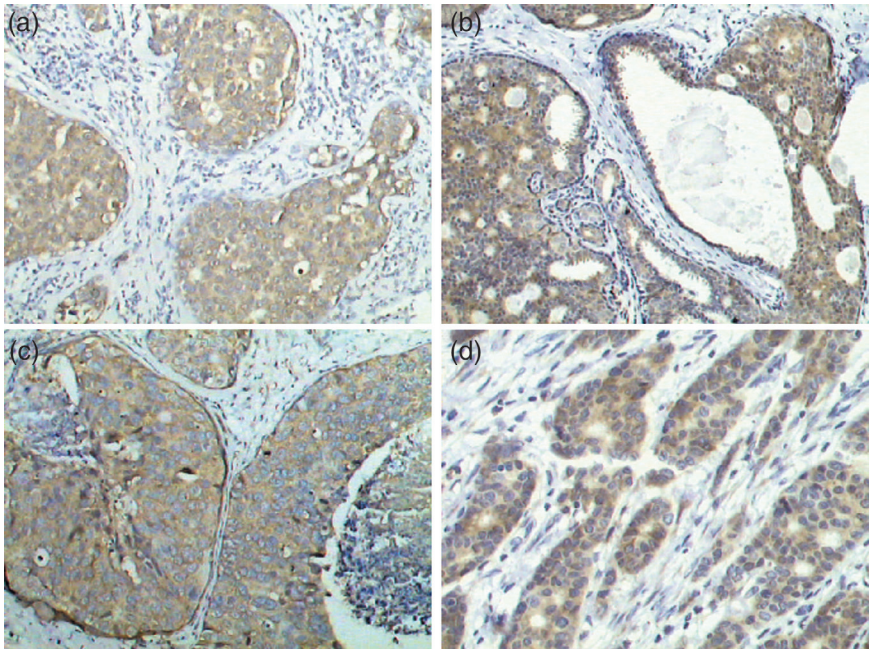

Fig. 4. The expression of α-tubulin protein in the various lesions of the breast. (a) Positive expression (+ +) of α-tubulin protein observed as brown-yellow color in the cytoplasm in atypical ductal hyperplasia (ADH)/peripheral papilloma with atypical hyperplasia (PPAH) (Immunohistochemistry-Streptaviden Peroxidase (IHC-SP) method); (b) Positive expression (+ +) of α-tubulin protein observed as brown-yellow color in the cytoplasm in ADH/PPAH (IHC-SP method); (c) Positive expression (+ + +) of α-tubulin protein observed as brown-yellow color in the cytoplasm in ductal carcinoma *in situ* (IHC-SP method); (d) Positive expression (+ +) of α-tubulin protein observed as brown-yellow color in the cytoplasm in invasive ductal carcinoma (IHC-SP method).

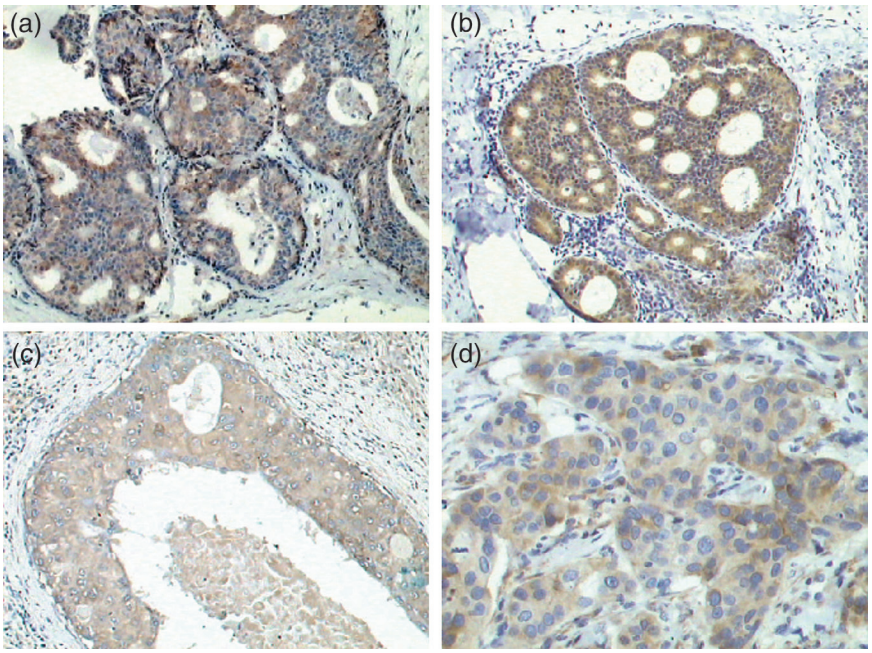

Fig. 5. The expression of γ-tubulin protein in the various lesions of the breast. (a) Positive expression (+ + +) of γ-tubulin protein observed as brown-yellow color in the cytoplasm in atypical ductal hyperplasia/peripheral papilloma with atypical hyperplasia (Immunohistochemistry-Streptaviden Peroxidase (IHC-SP) method); (b) Positive expression (+ + +) of γ-tubulin protein observed as brown-yellow color in the cytoplasm in ductal carcinoma *in situ* (DCIS) (IHC-SP method); (c) Positive expression (+ +) of γ-tubulin protein observed as brown-yellow color in the cytoplasm in DCIS (IHC-SP method); (d) Positive expression (+ +) of γ-tubulin protein observed as brown-yellow color in the cytoplasm in invasive ductal carcinoma (IHC-SP method).

Table 4. Centrosome abnormality in four groups

Groups in the histology	No.	Cases with centrosome abnormalities
NBT	60	0 (0%)
ADH/PPAH	80	24 (30%)
DCIS	80	42 (52.5%)
IDC	80	56 (70%)
Total	300	

Abbreviations: NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

Table 5. Mean values of DNA copy numbers of α-, γ-tubulin (× 10^6/ng)

Groups in the histology	Mean values of DNA copy numbers	
	α-tubulin	γ-tubulin
NBT	2.05	2.17
ADH/PPAH	4.31	4.86
DCIS	5.54	7.04
IDC	5.15	7.43

Abbreviations: NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

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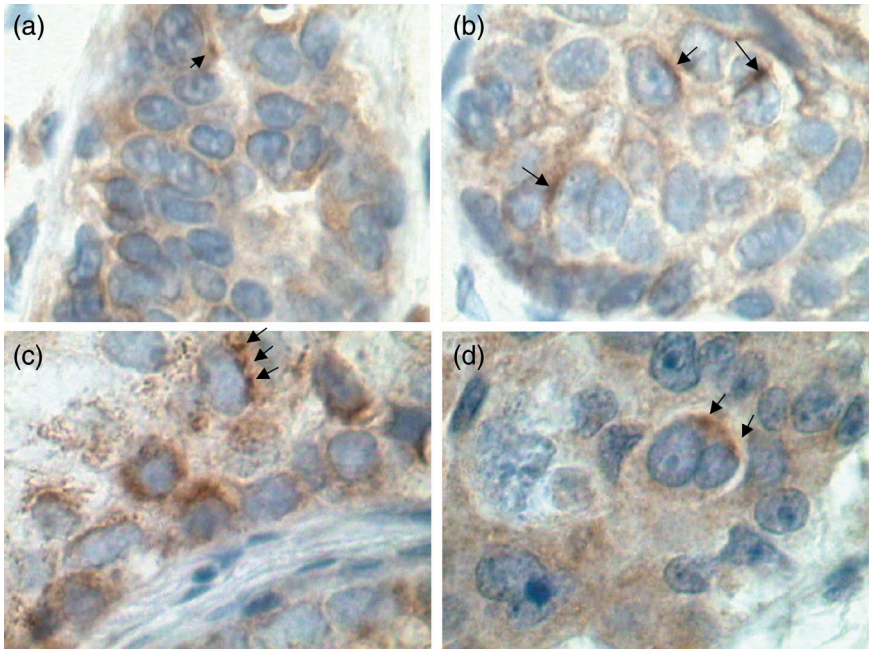

Fig. 6. Centrosome abnormality in the various lesions of the breast. (a) Normal centrosome (marked with an arrow) in normal breast tissue; (b) The elongated centrosome (marked with arrows) in atypical ductal hyperplasia; (c) The supernumerary centrosome (marked with arrows) in ductal carcinoma *in situ*; (d) The elongated centrosome (marked with arrows) in invasive ductal carcinoma.

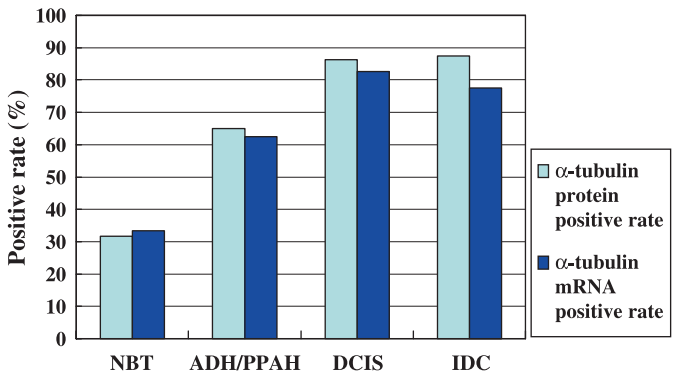

Fig. 7. Comparison of positive rate of α-tubulin messenger RNA (mRNA) and protein expression in each group. No significant differences were observed between mRNA and protein expression in normal breast tissue, atypical ductal hyperplasia/peripheral papilloma with atypical hyperplasia and ductal carcinoma *in situ*. In invasive ductal carcinoma, the mRNA expression was lower than protein expression, and the staining intensity showed statistically significant difference. NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

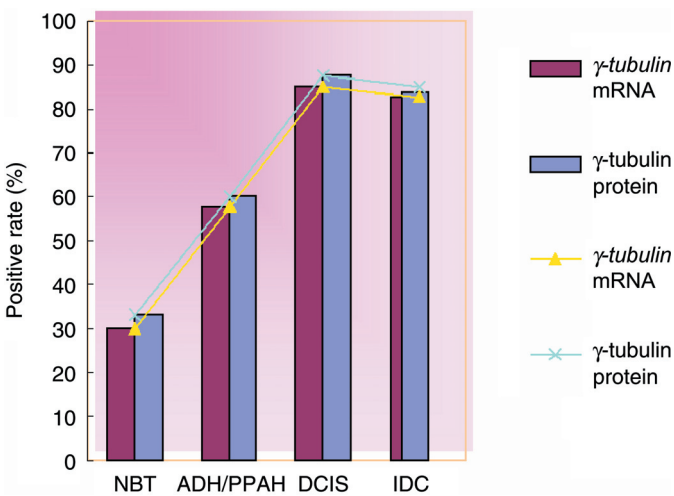

Fig. 8. Comparison of the positive rate of γ-tubulin messenger RNA (mRNA) and protein expression in each group. There were no significant differences between the positive rate of γ-tubulin mRNA and protein expression in any group. NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

The manifestation of centrosomal aberration in a wide range of tumor types was variable. What roles does the centrosome play in carcinogenesis? Although earlier studies indicated that centrosomal aberration was a late event in tumorogenesis, recent data suggest that centrosomal abnormality may be an early change and contribute significantly to cellular tumorogensis, and this warrants further investigation.[23]

Reports of assessing centrosomal abnormalities by profiling tubulin gene expression are few, and most of these evaluated tubulin protein expression.[24,25] Schneeweiss *et al*.[26] examined centrosomal protein expression in 103 primary invasive breast cancer tissues using IHC technique and found that centrosomal aberrations were associated with the factors predicting a more aggressive course of disease. In this study, we investigated centrosomal abnormality in the sequence of NBT, ADH/PPAH, DCIS and IDC by assessing α and γ-tubulins at the DNA, mRNA transcription and protein expression levels, and γ-tubulin antibody specifically labeled centrosomes by immunohistochemical staining technique in paraffin-embedded tissue sections was used for validation. A new method of PCR quantification, real time quantitative PCR, was performed because it allows us to actually view the increase in the amount of DNA. Although fluorescent *in situ* hybridization (FISH) is an established technique for determining gene amplification, qPCR represents an alternative method and has been shown to be a reproducible technique given the automation, sensitivity, specificity and quickness of this technique. In some studies for detecting HER-2/neu gene amplification,[27,28] good concordance was found between qPCR and FISH, especially for microdissected areas of paraffin-embedded breast carcinoma samples. qPCR not only could reach the accuracy of FISH, but also it has great advantages: it is high-throughput and fully automated, and can be relatively simply performed.

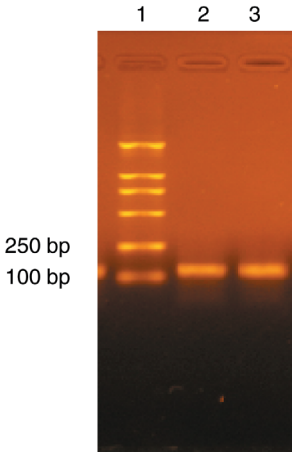

Fig. 9. Electrophoregram for quantitative polymerase chain reaction (qPCR) amplification products of *α-tubulin*. Lane 1: DL-2000 DNA Marker, Lane 2 and Lane 3 qPCR products of *α-tubulin*. One bright band was displayed above 100 bp.

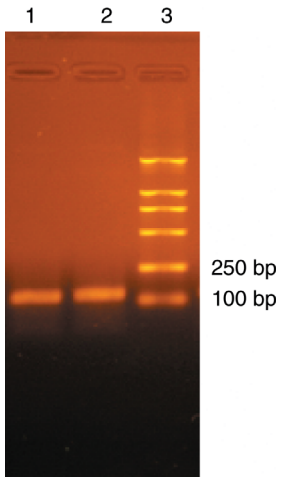

Fig. 10. Electrophoregram for quantitative polymerase chain reaction (qPCR) amplification products of γ-tubulin. Lane 1: DL-2000 DNA marker, Lane 2 and Lane 3 qPCR products of γ-tubulin. One bright band was displayed above 100 bp.

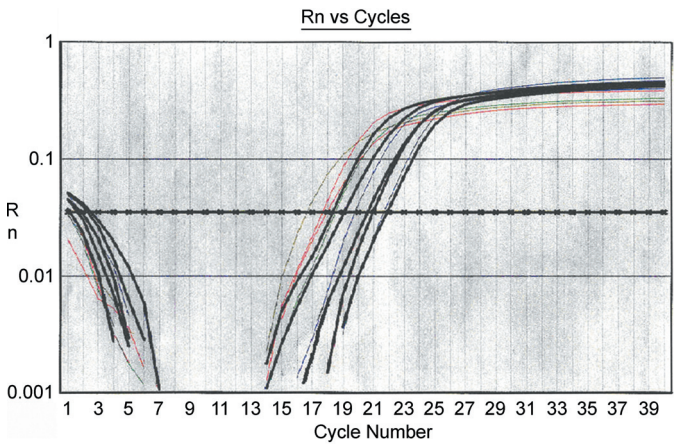

Fig. 11. Quantitation data of the external control gene system represented by fluorescent intensity curves of the standard during polymerase chain reaction amplification.

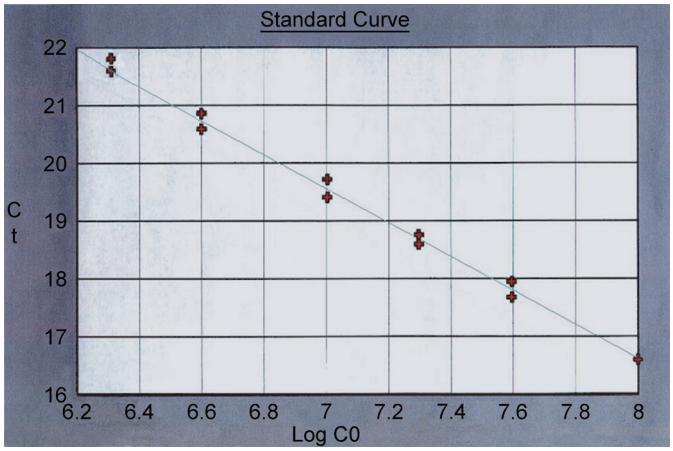

Fig. 12. Standard curve constructed from six known concentrations (copy number range = $1 \times 10^8/\mu L - 2 \times 10^6/\mu L$).

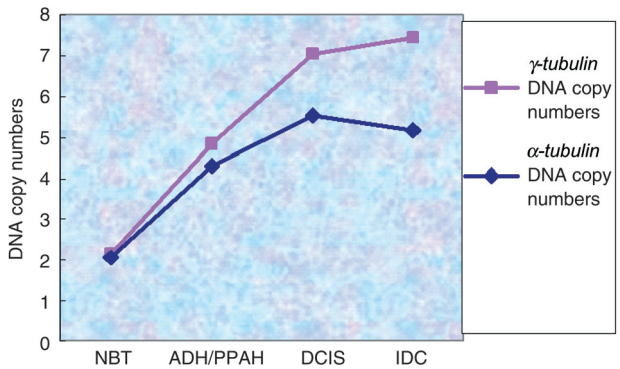

Fig. 13. Comparison for variation trend of DNA copy numbers of α, *γ-tubulin* in four groups. NBT, normal breast tissue; ADH, atypical ductal hyperplasia; PPAH, peripheral papilloma with atypical hyperplasia; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

Our results showed that the expression of α and *γ-tubulin* mRNA was concordant with that of protein; both of them were found to be increased from NBT to ADH/PPAH to DCIS and IDC, with DCIS and IDC showing similar, highest levels, thus suggesting that centrosomal aberration was characteristic of breast cancer. Although centrosome protein overexpression is frequently reported in human cancers, the studies of the molecular mechanisms responsible for this are few. It was reported that the over-expression of α, γ-tubulin proteins may be derived from mRNA over-expression and gene amplification.[29] However, in IDC, in comparison to DCIS, α, γ-tubulin protein expression continued to increase, but mRNA expression decreased slightly, suggesting that mechanisms other than mRNA over-expression, such as activating mutation of certain oncogenic and tumor-suppressor proteins may also contribute to centrosome protein over-expression.[30] Mutations of oncogene and tumor-suppressor gene disrupt the normal function and integrity of centrosomes and directly induce centrosome protein overexpression, thus resulting in chromosome instability.

In the current study, the mRNA expression of α and *γ-tubulin* in IDC was lower than DCIS. This may imply that cell divisions were most active in the evolution of precancerous lesions into DCIS. Centrosomal alterations increased dramatically at the onset of *in situ* carcinoma and subsequently fell when cancer cells invade into the stroma.

Our results demonstrated that DNA copy numbers of *γ-tubulin* were significantly higher in DCIS and IDC than in NBT and

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ADH/PPAH, and these were in accordance with the mRNA and protein expression profiles. As the DNA copy numbers of *γ-tubulin* increased from NBT to ADH/PPAH, DCIS to IDC, *γ-tubulin* gene amplification may play an important role in the genesis and development of breast cancer and it may be suggested that DNA copy numbers of *γ-tubulin* may be used as a potential indicator for the malignant transformation of breast lesions and the aggressive clinical behaviors of breast cancer in the future.[31,32] This could be testified by further study of *γ-tubulin* in a large cohort of breast cancer patients with complete follow-up data. Our results also showed that *γ-tubulin* gene amplification were consistent with mRNA over-expression, which suggest that the gene amplification might lead to mRNA over-expression. Since there was discrepancy for *α-tubulin* and *γ-tubulin* gene amplification, a number of interesting issues were left unanswered. *γ-tubulin* is distributed throughout the Pericentriolar Material (PCM) and exists in a ring-shaped multiprotein complex, known as *γ-tubulin* ring complex (γTuRC) that mediates centrosome microtubule nucleation. In contrast, *α-tubulin* is one of components of centrioles.[6] So, the different mechanisms of *α-tubulin* and *γ-tubulin* involved in breast tumergenesis need to be further investigated.

Accumulating evidence suggests that chromosomal instability is an early event and can induce malignant transformation, and the centrosome is a key organelle that controls chromosome segregation in cell division. Whether centrosomal aberrations are simply a consequence of chromosomal instability or a primary cause for carcinogenesis remains an open question. Our results demonstrated that centrosomal over-expression and amplification presented in premalignant lesions and *in situ* carcinoma may represent a very early event in breast cancer development.

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