The Immuno-fluorescence Quantity Analysis of $\alpha$-Tubulin and $\gamma$-Tubulin Protein in Precancerous Lesion and Carcinoma of the Breast and Its Significance

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OBJECTIVE To investigate the changes and values of the expression of $\alpha$-tubulin and $\gamma$-tubulin in atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) of the breast. The relationship between centrosome abnormalities and breast tumor development was further discussed.

METHODS There were three groups including ADH, DCIS and IDC with 30 cases in each group. They were analyzed by immuno-fluorescence quantity analysis. The expression levels of $\alpha$-tubulin and $\gamma$-tubulin protein in these tissues were detected by flow cytometry immuno-fluorescence analysis and compared with the results from normal tissues. Immunohistochemistry was also performed in this research.

RESULTS The results showed significant differences of the average of the positive (FITC labeled) cells ($P=0.000$) among the four groups. The level of the IDC group was the highest, while normal breast tissue showed the lowest level. The results suggested that the expression levels of $\alpha$-tubulin and $\gamma$-tubulin both increased as the grade of cellular proliferation and differentiation increased. The expressions showed significant differences among all the groups, except between the ADH and DCIS. There were no significant differences between $\alpha$-tubulin and $\gamma$-tubulin expression in each group ($P<0.05$), as there was agreement in the immuno-fluorescence and immunohistochemical analysis for protein expression.

CONCLUSION There is abnormal expression of centrosome tubulin as an early event in the development of breast tumor. Furthermore these aberrations may play a key role during oncogenesis and promote cellular transformation to malignancy. The immuno-fluorescence quantitative analysis and immunohistochemistry can complement each other.

KEYWORDS: centrosome $\alpha$-tubulin protein, centrosome $\gamma$-tubulin protein, precancerous lesion, breast carcinoma, immuno-fluorescence quantity analysis.

Introduction

The centrosome$^{[1]}$ is the major microtubule organizing center (MOTC) in animal (mammalian) cells. In recent years, foreign scientists$^{[2]}$ have discovered the centrosomal abnormalities in many kinds of cancer cells, indicating that it may relate to tumorigenesis. On the other hand there are few articles$^{[3,4]}$ concerning the relationship between breast cancer and the centrosome abnormalities. In this study the mechanism of the occurrence of breast cancer was explored. Flow cytometry immuno-fluorescence quantitative analysis was used to test the abnormalities of $\alpha$-tubulin and $\gamma$-tubulin in the centrosome.
Materials and Methods

Samples
The samples were collected in the Breast Pathological Department and Research Laboratory of the Cancer Institute and Hospital, Tianjin Medical University during the period from August, 2003, to October, 2004. These samples were obtained from the breast excision surgeries taken from the all the clinical materials. All of the paraffin blocks and pathological sections were reviewed and diagnosed by two pathologists following the WHO Histopathologic Classification of Breast Tumors. The 30 cases of atypical ductal hyperplasia (ADH) included atypical ductal epithelial hyperplasia and peripheral papillomatosis with atypia. The groups of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), which had the same number of cases were also selected randomly. The detailed clinical and pathologic data of the samples such as ages, TNM stage, pathology grades and axillary lymph node status were recorded. The normal control group was composed of 30 cases who had undergone breast segmentectomy. In addition, a number of immunohistochemistry tests were performed on the specimens obtained from the same hospital during January, 1998 to January, 2004. The samples were selected randomly and separated into four groups: 40 cases of atypical hyperplasia, 40 cases of invasive ductal carcinoma, 40 cases of DCIS, and 30 normal tissues as the control. These results were compared.

Agents and equipment
The following agents were purchased from the Beijing Zhongshan Biotechnology Co.: mouse anti-human α-tubulin monoclonal antibody (Zymed Co.), mouse anti-human γ-tubulin monoclonal antibody (Santa Cruz Co.), mouse anti-human p53 monoclonal antibody IgG1, FITC-conjugated sheep anti-mouse immunoglobulins, histostainTM-SP (Zymed Co.), PBS buffer (pH 7.2–7.4), Tween-20 and paraformaldehyde were brought from the Tianjin Chemical Reagent Co.

A flow cytometer (Coulter EPICS-XL) and automatic immunohistochemistry (Biogenex AS60302) were used in the study.

Experimental methods
Flows cytometry immuno-fluorescence analysis
When the centrosome proteins were conjugated with anti-α-tubulin antibody (or γ-tubulin antibody)-IgG-FITC, the expression of fluorescence intensity from the centrosome proteins were determined by flow cytometry. The fluorescence or light from FITC labeled cells, scattered into all orientations when the cells passed through the laser area. The light was detected by an optical lens and then converted into an electrical pulse signal by detectors (mainly PMTs). The signal was processed and amplified. By this method various types of parameters, graphics, and statistics were obtained.
Immunohistochemistry
The positive signals of α-tubulin and γ-tubulin were located in the epithelial cytoplasm showing a brown-yellow color. According to the quantitative scoring system improved by Tanaka et al. [8], the stainings were categorized into four ranks as follows: 0 rank (0–1); 1 rank (2–4); 2 rank (5–8); and 3 rank (9–12).

Statistical analysis
The data were analyzed by the SPSS 10.0 software package. The differences among the groups were compared by analysis of variance (F test), Newman-Keuls test (q test) and t test. P<0.05 was considered to be significant.

Results

Anti–α-tubulin flow cytometry immuno-fluorescence analysis
The centrosome protein in cytoplasm was conjugated with anti-α-tubulin antibody-IgG-FITC. The cells which expressed excessively centrosome protein were labeled green fluorescence. α-Tubulin FITC-labeled positive cells rates are shown in Fig.1 and Fig.2. The percent of FITC-labeled cells in every group is shown in Table 1. The average rates of the α-tubulin FITC-labeled positive cells were from 3.18 in the group with the normal tissues to 23.09 in the group with the IDC. The average rates were obviously raised following cell hyperplasia and the development of cancer.

Table 1. Average rate of the FITC-labeled positive cell.

| Groups | α-Tubulin | | | | | γ-Tubulin | | | |
|--------|-----------|-----------|-----|-----|--------|-----------|-----------|-----|-----|--------|-----------|
|        | FICT-cell rate (%) | Mean | Cases | FICT-cell rate (%) | Mean | Cases |
| Normal tissue | 0.19–6.60 | 3.18 | 30 | 1.12–7.20 | 3.33 | 30 |
| ADH | 0.45–17.72 | 11.58 | 30 | 0.95–17.40 | 10.67 | 30 |
| DCIS | 4.72–44.30 | 14.84 | 30 | 5.29–33.15 | 14.45 | 30 |
| IDC | 4.93–62.33 | 23.09 | 30 | 7.55–62.36 | 24.49 | 30 |
| Total | 0.19–62.33 | 13.17 | 120 | 0.95–62.36 | 14.04 | 120 |

Anti–γ-tubulin flow cytometry immuno-fluorescence analysis
Like the result with α-tubulin, over-expressed γ-tubulin was also manifested by green fluorescence labeling, as shown in Fig.3 and Fig.4. The percent of FITC-labeled cells in every group is shown in Table 1. Similarly, the average rates of the FITC-labeled positive cells for γ-tubulin were from 3.33 in the normal tissue group to 24.49 in the IDC group. The average rates were increased significantly following cell hyperplasia and the development of cancer.

Table 2. Expression of anti-α-tubulin and anti-γ-tubulin.

<table>
<thead>
<tr>
<th>Groups</th>
<th>α-Tubulin average score (range)</th>
<th>Cases</th>
<th>γ-Tubulin average score (range)</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>1.07 (0–4)</td>
<td>30</td>
<td>1.20 (0–5)</td>
<td>30</td>
</tr>
<tr>
<td>ADH</td>
<td>3.18 (0–10)</td>
<td>40</td>
<td>3.85 (0–11)</td>
<td>40</td>
</tr>
<tr>
<td>DCIS</td>
<td>6.05 (0–12)</td>
<td>40</td>
<td>5.63 (0–12)</td>
<td>40</td>
</tr>
<tr>
<td>IDC</td>
<td>7.02 (0–12)</td>
<td>40</td>
<td>6.40 (0–12)</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>4.53 (0–4)</td>
<td>150</td>
<td>4.17 (0–5)</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 3. Comparison of FITC-labeled positive cell average rate among four groups.

<table>
<thead>
<tr>
<th>α-Tubulin</th>
<th>γ-Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>F=26.723</td>
<td>P=0.000</td>
</tr>
<tr>
<td>F=32.932</td>
<td>P=0.000</td>
</tr>
</tbody>
</table>

Comparison of 4 groups

| Normal/ADH | q=8.398 | P=0.000 |
| Normal/DCIS | q=11.659 | P=0.000 |
| Normal/IDC | q=11.186 | P=0.000 |

Comparison between any two groups

| Normal/IDC | q=19.907 | P=0.000 |
| ADH/DCIS | q=3.261 | P>0.05 |
| ADH/IDC | q=11.504 | P=0.000 |
| DCIS/IDC | q=8.258 | P=0.000 |

Expression of anti-α-tubulin and γ-tubulin by immunohistochemistry
The immunohistochemical staining of α-tubulin and γ-tubulin developed in the cytoplasmic compartment in part of the samples with both ductal epithelial cells and cancer cells. Positive cells were shown to be granular at different intensities with the background being clear. The positive scores and expression of α-tubulin and γ-tubulin in different groups are shown on Table 2. The average score of α-tubulin expression or the average score of γ-tubulin were both increased by degrees compared to the group with the normal tissues and the IDC group.
Statistical analysis results

The percentages of cells over-expressing α-tubulin were different among these groups, according to the variance analysis ($F=26.723$, $P=0.000$), suggesting there were significant relations between the degree of the cellular hyperplasia and the cancer cell growth or differentiation. The abnormalities of centrosome protein were directly proportional to the percentage of abnormal cells. By doing a $q$ test (Newman-Keuls test) on the average of the four groups it was concluded that the percent of positive cells in the control was less than those in the ADH, DCIS and IDC groups, all of which were $P<0.05$. Furthermore the number of positive cells in the IDC group was larger than in DCIS and ADH, $P<0.05$. However, there was no significant difference between ADH and DCIS, $P>0.05$.

The results of the statistical analysis are shown in Table 3.

A similar result was displayed in γ-tubulin by analysis of variance. The $q$ test also showed there was no correlation between DCIS and ADH, $P>0.05$.

The statistical analysis of the immunohistochemical results: the expression of α-tubulin was significantly different among the four groups, $F=27.653$, $P=0.000$, as was the case for γ-tubulin ($F=22.850$, $P=0.000$). The comparison between any two groups also showed significant differences, except for those between the DCIS and IDC groups, $P>0.05$.

The percentages of positive cells expressing α-tubulin and expressing γ-tubulin in every group were compared using the t-test. There was no significant differences among all of them. $t=0.669$, $P=0.278$; $t=0.185$, $P=0.619$; $t=0.383$, $P=0.492$, $P>0.05$.

The immunofluorescence results analyzed by a dispersed dot plot were consistent with the immunohistochemical results. The main ascending line gave a positive correlation (Fig.7).
The centrosome is an important part of the cytoskeleton. It consists of a pair of centrioles and pericentriolar material (PCM). Centrioles, composed of α-tubulin, play a fundamental role in centrosome stability and centriole duplication.[9] The PCM, which contains an amount of useful components such as γ-tubulin is the site of microtubulin nucleation. The normal centrosome[10] influences many important events in the cell cycle, especially in mediating moving of the mitosis spindle, aiding in high fidelity of chromosome separation, and the stabilization of the genome in daughter cells. Currently, the abnormalities of the centrosome have been observed[11,12] in a wide range of malignant tumors. Therefore, the centrosome has become the focus of research in the field of tumor cell biology and molecular pathology.

Breast cancer is one of the most common malignant tumors in females, having a pathogenesis that is complex and not clear[13]. It was reported that abnormal expression of centrosome proteins was detected in breast invasive carcinoma by German scientists in 2003[14]. This discovery motivated our team to investigate the changes in the centrosome and its function at an early stage of breast cancer and precancerosis. We have investigated the mechanism of tumorgenesis by studying the following conditions: DCIS, the most common breast carcinoma in situ, ADH, a known precancerous lesion, and IDC, the most frequently occurring invasive carcinoma. Normal tissue served as the control.

With regard to the numbers of FITC positive cells labeled as α-tubulin and γ-tubulin in normal tissue, the following tissues, ADH, DCIS and IDC, were significantly different based on flow cytometric testing. The number of α-tubulin and γ-tubulin positive cells in IDC was greater than in normal tissue, and a similar result was also obtained by immunohistochemistry, supporting the conclusion that a centrosome abnormality is one of the important features in breast cancer cells. In further experiments, the number of positive cells in ADH was much greater than that in normal tissue, but there was no significant difference between ADH and DCIS. This showed that over-

### Discussion

The centrosome is an important part of the cytoskeleton. It consists of a pair of centrioles and pericentriolar material (PCM). Centrioles, composed of α-tubulin, play a fundamental role in centrosome stability and centriole duplication[9]. The PCM, which contains an amount of useful components such as γ-tubulin is the site of microtubulin nucleation. The normal centrosome[10] influences many important events in the cell cycle, especially in mediating moving of the mitosis spindle, aiding in high fidelity of chromosome separation, and the stabilization of the genome in daughter cells. Currently, the abnormalities of the centrosome have been observed[11,12] in a wide range of malignant tumors. Therefore, the centrosome has become the focus of research in the field of tumor cell biology and molecular pathology.

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![Fig. 5. Positive expression of α-tubulin in ADH (IHC S-P×100).](image)

![Fig. 6. Positive expression of γ-tubulin in DCIS (IHC S-P×100).](image)

![Fig. 7. Correlation of α-tubulin by using immunofluorescence and immunohistochemistry.](image)
expression of centrosome proteins was present in both precancerosis and DCIS, supporting the hypothesis that centrosome abnormalities are an early event in breast tumorgenesis[15,16]. Centrosome defects lead to cell over-multiplication and deterioration, causing genomic instability. DCIS, ADH, and IDC are associated with fully developed breast cancer.

The immuno-fluorescence results were consistent with the immunohistochemical results. But when any two groups were compared separately, the results did not show consistency. Perhaps different methods can produce different results. Flow cytometry testing can measure the quantity of α-tubulin or γ-tubulin expression by counting positive cells. In this case, the results are more objective. Immunohistochemistry is a proven qualitative and semi-quantitative method which is easy and feasible for clinical laboratory examinations. But it is difficult to judge the result objectively. Using two methods helps to get an objective result. Thus, it is better to use two combined methods for mutually complementary verification.

References

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