The Role of Angiogenesis in Breast Atypical Hyperplasia and Breast Carcinoma and It´s Correlations with PCNA, p53 and c–erbB–2

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Abstract **Objective** To investigate the role of angiogenesis in breast atypical hyperplasia and breast carcinoma and its correlations with PCNA, p53 and c-erbB-2. **Method** Angiogenesis was analyzed in 10 normal breasts, 20 cases of simple hyperplasia, 22 cases of mild atypical hyperplasia, 21 moderate or severe atypical hyperplasia as well as 30 breast carcinoma by immunohistochemical method. Microvessel endothelial area(MEA), which were stained by anti-Factor Ⅷ-related antigen(FⅧ-RA) polyclonal antibody, were quantitatively assessed by Computer-Aided Image Analysis System(CIAS). p53 oncogene protein, c-erbB-2 oncogene protein and Proliferating Cell Nuclear Antigen(PCNA) in these lesions were detected by immunohistochemical method. The relationships of MEA with hyperplasia severity, PCNA- LI, p53 oncogene protein and c-erbB-2 oncogene protein were analyzed. **Results** (1) MEAs were significantly different(P<0.05) among mild atypical hyperplasia, moderate or severe atypical hyperplasia and malignancy.(2) MEA was positively associated with PCNA-LI(r=0.5416, P<0.001) in these lesions. (3) The expression of p53 oncogene protein instead of c-erbB-2 was correlated with angiogenesis in breast atypical hyperplasia and carcinoma(P<0.005). **Conclusions** Neovascularization occurs in breast hyperplasia, especially in atypical hyperplasia, in the meantime angiogenesis may speculate upon the malignant risk of breast precancerous lesion, associated with cell proliferative activity and p53 oncogene protein.

Key Words breast atypical hyperplasia; breast carcinoma FⅧ-RA; p53 oncogene protein; c-erbB-2 oncogene protein; proliferation cell nuclear antigen

It was well known that the risk of carcinogenesis in breast hyperplasia was higher than that in normal mammary gland and the possibility of canceration varied with the severity of atypical hyperplasia[1, 2]. In the multistep process of malignancy, angiogenesis behaves as an event independent and important factor of the other pathways of tumor progression[4,5,6]. The hypothesis that tumor growth depends on the induction of neovascularization originated in the 1960s from experiments in which tumor grew in isolated perfused organ[4,6]. Recently this relationship has been well established[7]. Experimental and clinical data indicate that most tumors arise from precancerous lesions such as atypical hyperplasias and induce neovascularization when a subset of cells within the tumor switches to the angiogenic phenotype. The switch to angiogenic phenotype may occur before the full development of tumor, i.e. in the preneoplastic or the early stage of carcinoma[8]. Previous experimental studies had suggested that angiogenesis could be an indicator of a high risk of breast cancer in woman with fibrocystic hyperplasia of breast[9]. The purpose of this study was to assess the microvessel endothelial area (MEA) condition of stromal microvessels in atypical hyperplasia of breast and breast carcinoma, which was quantitated by Computer Aided Analysis System, and to investigate the relationships between MEA and atypical hyperplasia severity, p53 and c-erbB-2 oncogene protein expression and cell proliferating activity.

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MATERIALS AND METHODS

1998 in the first Affiliated Hospital of Chongqing Medical University. The cases with simple epithelial hyperplasia, mild atypical hyperplasia, moderate or severe atypical hyperplasia and breast carcinoma were 20, 22, 21 and 30 respectively. Samples of 10 specimens with normal breasts were selected from hypermastia patients who underwent mastoplasty treatment and were diagnosticated by pathologist. According to the morphologi-

cal criteria[1], benign lesions were classified into simple epithelial hyperplasia: there are 3 or 4 cell layers above a basement membrane and the epithelial hyperplasia is normal type without atypical; mild atypical hyperplasia: there are more than 4 cell layers above a basement membrane and the epithelial cells have slight atypia and tend to cross and distend spaces; moderate or severe atypical hyperplasia: the cavities of enlarged ductal or lobular glands were filled with different morphologic cells and the cellular appearance is obvious heteromorphic and atypical. Tissue from each case was processed routinely, fixed in 10% neutral buffered formalin, and embedded in paraffin. The archived blocks were in series sectioned at a thickness of 5μm. One of them stained with hematoxylin and eosin(H.E.) was histopathologically assessed. Others remained sections for immunohistochemical staining were toasted 1 hour at 56℃, then kept in drying oven at 37℃.

Immunohistochemical Determinations

Blood Vessel Immunohistochemistry

Vessel was stained with standard avidin-biotin-complex technique. Briefly 5μm sections were deparaffinized in 50% pure dimethylbenzene, dehydrated through 100%, 95%, 80%, 70% series ethanol, quenched in 1% methanol H_2O_2 10 minutes, after digestion in 0. 4% pepsin solution at 37 ℃ for 30 minutes, covered with 5% normal goat serum at 37℃ for 20 minutes, polyclonal rabbit antibodies to human Von Willebrand Factor(Factor Ⅷ) (Zymed, America) were applied to the sections at a dilution of 1:100 overnight at 4℃. After being washed, the sections were incubated with biotinylated goat antibodies to rabbit immunoglobulins for 20 minutes at 37℃. Following washing with PBS 3 times, an avidin-biotin-peroxidase complex was applied to the sections according to the manufacturers specifications(DAKO, 1:100). The slides were developed using diaminobenzidine to produce a brown reaction product under controll and were lightly counterstained with hematoxylin. Microvessel endothelial area (MEA) and methodolgy of quantitation MEA was determined by Computor-Aided Image Analysis System (Leica, MD20 Germany). Hot spots(most vascular areas in sections) were found by scanning the sections at low power(10×and 25.6×) and identifying those areas having the greatest number of anti-FⅧ-RA-stained microvessels per area, then the selected hot spot was switched into CIAS via true color video tube, for automatic detection of the brown-stained endothelial cells of the vessels using a chromaticity-based color analysis. Vessels with muscular wall and vascular lumen bigger than eight erythrocytes were excluded on the screen of computer. Finally the stained MEAs were quantitated. Five hot spots were detected at each section and finally MEA(μm) was the mean of five times.

Proliferating Cell Nuclear Antigen(PCNA)

The 5 μm thickness sections were dewaxed in xylem and rehydrated in decreasing concentrations of ethanol. The endogenenous peroxidase activity was blocked with 1% methanol hydrogen peroxide for 10 minutes. The sections were heated in PH 6.0 citrate PBS at 92℃ for 10 minutes and got cold at room temperature to reduce nonspecific antibody binding. Immunostaining was performed using a mouse monoclonal anti-human-PCNA antibody(PC10, DAKO) diluted at 1: 100 for 24h at 4℃. Antibody binding was visualized by the streptavidin-biotin-immunoperoxidase method (DAKO, 1: 100), followed by 0.05% diaminobenzidine dovelopment. Sections were counterstained with hematoxylin. A PCNA positive breast carcinoma served as positive control. Anti-human-PCNA antibody was replaced with PBS in negative control. According to Steven criteria[7], cells were considered to be positive for PCNA if there was strong brown granular or confluent staining of the nucleoplasm and nucleoli. The PCNA-labeling index(PCNA-LI) by PCNA staining was evaluated by counting 100 consecutive cells in one relatively high degree part of staining. Five parts were counted in each slide. Finally PCNA-LI was the mean of five times.

p53

After routine deparaffinization, the sections were blocked with 1% methanl H_2O_2. The slides were heated in citrate PBS (pH 6.0) at 92℃ for 10 minutes and got cold at room temperature, followed slowly washing in distilled water. The immunostaining of p53 was performed using a mouse monoclonal anti-p53 antibody (DAKO) diluted at 1:100, 24h at 4℃. The remaining steps for immunostaining were the same as described earlier. Positive control was the sections of breast carcinoma known to have p53 gene mutations and p53 protein accumulation. In the negative control group, the primary antibody was changed into PBS. Standard of Vermeulen[8] was utilized to evaluate the p53 protein staining. Positive "+" was scored if more than 10 percent of cells were positivly stained in 500 consecutive counted cells.

c–erbB–2

Staining for the c-erbB-2 protein was performed using

the rabbit polyclonal anti-human c-erbB-2 protein antibody(DAKO) diluted at 1: 100 for 24h at 4℃, using the same method as for the p53 protein. Positive controls included breast carcinoma known to exhibit high levels of c-erbB-2 protein. Negative controls were obtained with PBS instead of primary antibody. For c-erbB-2 protein expression, membrane or cytoplasm staining was considered positive. The degree of expression was scored as "+" for 10%-50% of cells staining and "++" for more than 50 % of cells.

Statistical Methods

MEA was calculated in the means ± standard deviation(x±s). One-Way analysis of variance(ANOVA), linear regression model and student test were used. Statistical analysis was performed using the SAS statistical software program(SAS Institute Inc. Cary. N.C.). 0.05 considered statistically significant.

RESULTS

MEA in Breast Atypical Hyperplasia and Carcinoma.

In all sections, FⅧ-RA immunoreactivity was confined to endothelial cell(Fig1, 2). Neovascular hot spots were generally found at the periphery stroma of the cancer nests and the stroma around the active proliferating epithelial islands. The means ± standard deviation of MEA by CIAS in normal breast, simple epithelial hyperplasia, mild atypical hyperplasia, moderate or severe atypical hyperplasia and breast carcinomas increased one by one (P<0.01). The differences among normal tissue, simple epithelial hyperplasia and mild atypical hyperplasia were not significant. Beside that there were significant difference between every two groups (P<0.05), (Table 1)

PCNA–LI vs. MEA

PCNA-LIs in normal mammary tissue, simple ep-

Table 1 MEA of Breast Atypical Hyperplasia and Carcinoma

	Sample	$\bar{x}\pm s\mu$(m2)	Range	P
Normal mammary	10	2249.81±792.4266	652.54-3230.73	>0.05
SEH	20	2932.90±1376.2400	1403.02-5181.08	>0.05
MAH	22	3315.33±762.3400	2210.64-4632.36	<0.05
SAH	21	4486.79±1382.4600	1646.66-6986.16	<0.01
BC	30	5568.31±1510.2600	3093.53-7873.08	

Note: Value represent the means ± standard deviation (SEH, simple epithelial hyperplasia. MAH, mild atypical hyperplasia. SAH, moderate or severe atypical hyperplasia. BC, breast carcinoma).

Table 2 Relationship Between p53 Protein and MEA in Breast Atypical Hyperplasia and Carcinoma

	MEA($\bar{X}\pm S$)		t	p
	p53+	p53-		
ormal	-	2249.81±792.4266	-	-
SEH	3454.46	3220.15±1469.5351	-	-
MAH	4194.89±498.5998	3216.94±803.9394	2.3024	<0.05
SAH	4525.82±1360.4827	3464.61±687.0646	2.1794	<0.05
BC	5278.02±1471.3515	5177.46±1355.3398	0.1926	>0.05

Table 3 Relationship Between c-erbB-2 Oncogene Protein and MEA in Breast Atypical Hyperplasia and Carcinoma

c-erbB-2	n	MEA($\bar{x}\pm s$)	F	P
-	49	3853.20±1301.41		
+	30	4041.72±1601.44	-0.5665	>0.05
++	24	4424.54±1204.49		

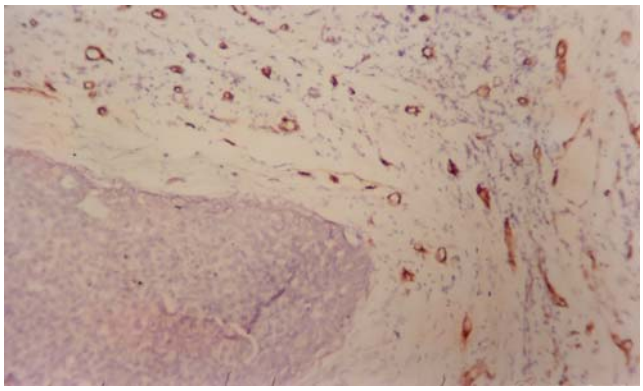

Fig 1 FⅧ-RA immunohistochemical stained in breast atypical hyperplasia ABC×100

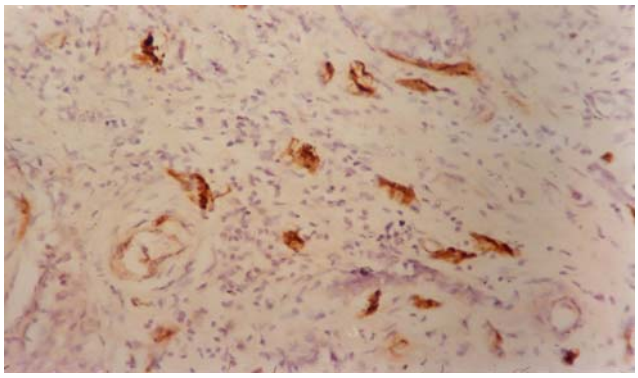

Fig 2 FⅧ-RA immunohistochemical stained in breast carcinoma ABC×100

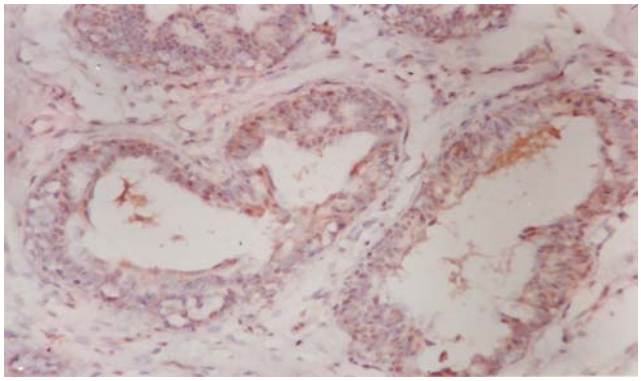

Fig 3 PCNA expressed in breast atypical hyperplasia ABC×100

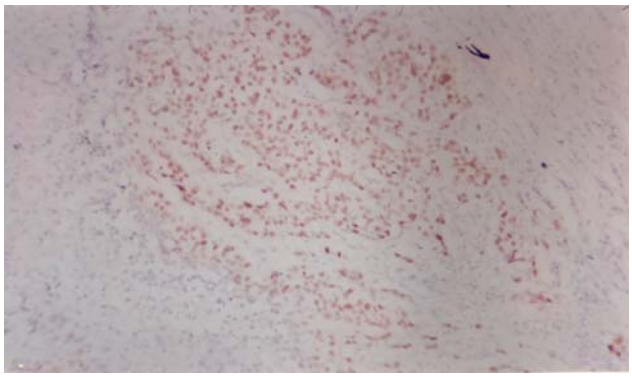

Fig 4 PCNA expressed in breast carcinoma ABC×100

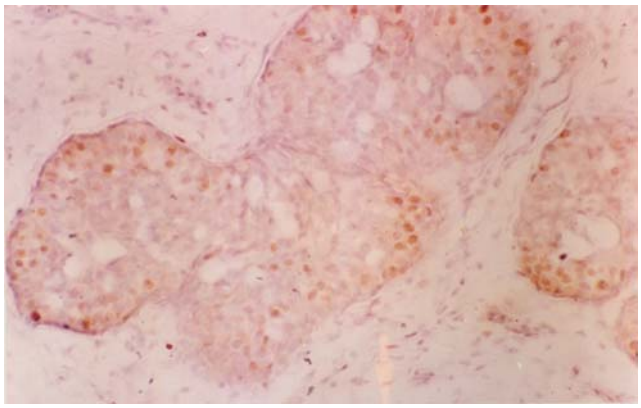

Fig 5 p53 gene protein expressed in breast atypical hyperplasia ABC×100

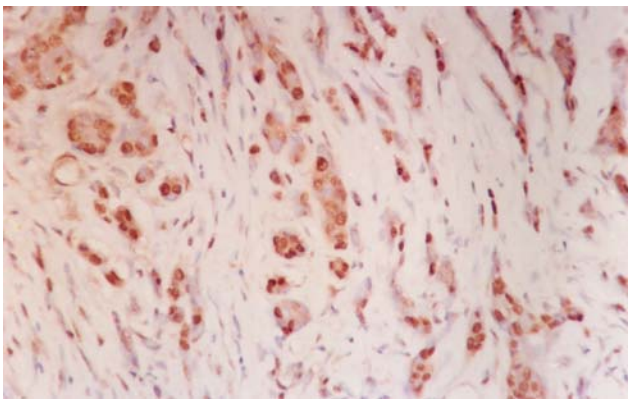

Fig 6 p53 gene protein expressed in carnoma ABC×100

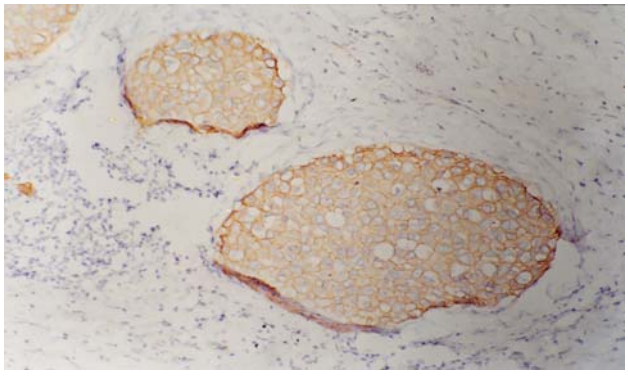

Fig 7 c-erbB-2 protein expressed in breast atypical hyperplasia ABC×100

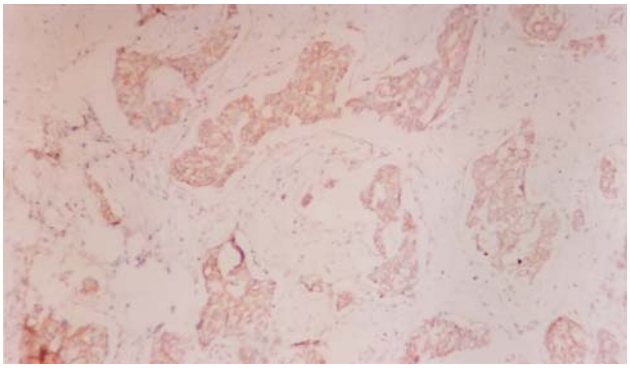

Fig 7 c-erbB-2 protein expressed in carnoma ABC×100

cant(r=0.5652, $P<0.001$).

p53 Oncogene Protein and MEA.

p53 oncogene protein expressed in benign and malignant tissue instead of normal mammary tissue. Expression rates in simple epithelial hyperplasia, mild atypical hyperplasia, moderate or severe atypical hyperplasia and carcinoma were 1/20 (5%), 4/22 (18.80%), 7/22(33.33%) and 12/30(40%), respectively(Fig5, 6). The relationship between p53 protein and MEA was shown in Table2. MEAs of p53 protein positive expression in mild and moderates or severe atypical hyperplasia were significantly higher than those of negative p53 protein expression lesion respectively ($P<0.05$, $P<0.01$)(Table 2).

c-erbB-2 Oncogene Protein and MEA (Fig7, 8)

With the c-erbB-2 oncogene protein expression becoming stronger and stronger, MEA increased slightly. But the difference was not significant ($P>0.05$)(Table 3)

DISCUSSION

Factor Ⅷ was an excellent vessel marker, labeling the endothelial cell surface as previously reported [6]. In breast cancer and precancerous lesion stroma, vessels outlines and sizes were extremely varible. When vessels were small and scattered within the tumor stroma, it was easy to count. But when vessels were larger, longer and crowded, the networks of vessel were difficult to individualize and count exactly. The distribution of vessels was also heterogeneous. So MEA of positive immunoreactions performed by Computer-Aided Image Analysis was deemed a more logical alternative for evaluation of F Ⅷ immunostained surface. In our study, we also excluded the vessels containing more than eight erythrocytes or with muscular wall in order to evaluate neovascularization accurately.

The results of this study indicate that neovascularization in normal mammary tissue to simple epithelial hyperplasia, mild atypical hyperplasia, moderate or severe atypical hyperplasia and breast carcinoma increased one by one. There was no significantly difference among the former three groups($P>0.05$). This result could be explained by that the degree of cell proliferating and metabolism were not significantly difference among them. MEA in moderate or severe atypical hyperplasia was higher than that in mild atypical hyperplasia lesion($P<0.05$) so as to satisfy the requirement for active growth and metabolism of proliferating mammary gland epithelial cells. Moreover, MEA in breast carcinoma was highest in these groups($P<0.01$). This result implied that there was strongly association between breast carcinogenesis and angiogenesis and the carcinogenesis progression depended on neovascularization. It was previously reported that angiogenesis status in rabbit iris, in which breast hyperplasia tissue was transplanted, was more obvious than that followed by transplantation with normal mammary tissue, and provided that the ability to induce angiogenesis could be used to identify tissues in which progression toward malignancy had already began[4]. Zich et al demonstrated that angiogenic capacity appeared long before a neoplastic transformation could be recognized and supported the hypothesis that acquisition of angiogenic capacity by a cell population normally devoid of this capacity indicated an increased risk of neoplastic transformation[11]. In a small cohort study of patients with fibrocystic breast disease who subsequently developed a histologically confirmed invasive breast carcinoma, Guinebretiere et al observed that the risk of developing breast cancer increased significantly with the density and concentration of intralesional microvessels[12]. Recently a series of studies published for angiogenesis in precancerous lesion of other organ tissue. Dellas et al observed that microvessel counts significantly increased in precancerous lesions and invasive cancer in cervix[8]. Dearing et al had acquired the same conclusion in prostate lesions[13]. Abulafia et al found that along with the degree of endometrial hyperplasia, the angiogenesis increased more and more[14]. Folkman reported that angiogenic activity first appeared in subset of hyperplasia islets before the onset of tumor formation, in which transgenic mice expressing an oncogene in the β-cells of the pancreatic islets heritably recapitulate a progression from normality to hyperplasia to neoplasia[5]. Feng and Liu suggested that abnormal hyperplastic vessels may be a prerequisite to cancerous transformation of nasopharyngeal epithelium[15]. In our study we consider that neovascularization occurs in breast hyperplasia, especially in atypical hyperplasia and the angiogenesis may speculate upon the risk of breast premalignant tendency to cancerous transfomation. The switch from avascular to the vascular phase is regulated by multiple biochemical and genetic mechanisms. Dameron et al showed that as normal cells underwent genetic changes that lead to malignancy they must switch from aninhibitory to an angiogenic phenotype[16]. There was paracrine regulation among malignant cells, stromal and vascular endothelial cells. Angiogenic peptides from malignant cells regulated neovascularization and vascular endothelial cells

secreted cytokines to stimulate malignant growth. Malignant cells communicate with neighboring stromal cells via a complex network of extracellular signals [17]. icrovascularization and blood flow within a tumor may be determinant factors of the tissue diffusion of anticancer agents. According to above mechanisms, therefore may be partly involved in responsiveness to therapy. Some drugs have antiangiogenic effect and were under clinical evaluation. On the other hand, those drugs were unlikely to be effective against established tumors [18]. Our study provided a possibility to inhibit or re-transformate the risk of breast premalignancy to malignancy by antiangiogenesis strategy.

There is different conclusion for the association between cell proliferating activity and angiogenesis. Smolle et al showed that there was a strong positive correlation between tumor proliferating activity and the number of small vessels(r=0.86, P<0. 001) at the base of melanocytic skin tumor and found that tumor cell lines had been shown to release substances which influence stroma and may induce vascular proliferation [19]. The same conclusion was identified in rectum and breast carcinoma[20]. Porschen et al found that angiogenesis influence tumor cell proliferating activity, and the tumor cell proliferation decreases with increasing distances from the blood vessel[21]. Although it was established that vessel endothelial cell proliferating activity in breast carcinoma was 45 times higher than that in benign lesion, Vartanian et al didn't find the relationship between tumor cell proliferating activity and angiogenesis[22]. In our study, PCNA-LI was positively correlated with MEA during the progression from normal mammary tissue, atypical hyperplasia to breast carcinoma(r=0.5652, P<0. 001). It showed that there was correlation between angiogenesis and cell proliferating activity. It was well known that tumor cells or non-malignant cells produce release angiogenic factors such as VEGF, bFGF[23, 24], which can stimulate their own proliferation as well as the proliferation of vascular endothelial cells. We suggested that along with the breast hyperplasia, especially atypical hyperplasia, epithelial cell has high proliferating activity for construction and growth metabolism and produces angiogenic factors to regulate the proliferating and prolonging of vessel endothelial cell. At the same time, endothelial cell could regulate the epithelial proliferation by paracrine bypass.

Nowadays some scholars were interested in the relationship between p53 oncogene protein and angiogenesis. Weidner et al reported that microvessel density was associated with p53 oncogene protein in 1993 [25] in head-and-neck squamous-cell carcinoma. This results was testified in colorectal carcinoma[26,27] and lung carcinoma[28,29]. These studies also have demonstrated that p53 tumor suppressor gene plays an important role in controlling tumor angiogenesis and there were more vascularization in tumor with p53 mutation than that in tumor without p53 gene alteration. Simultaneously, opposite results were concluded in non-small cell lung cancer and colorectal cancer[10,30]. Toi et al and Charpin et al did not find the correlation between angiogenesis and p53 protein in breast carcinoma [31,32]. In our study, we found that MEA in p53 oncogene protein positive expression lesions was significantly higher than that in p53 negative lesions in mild and moderate or severe atypical hyperplasia. This result indicated that p53 oncogene mutation, protein accumulation was associated with neovascularization in the period of carcinogenesis. No relationship was found between p53 protein and angiogenesis in breast carcinoma partially owing to the little samples of carcinoma and we will do further study for it. The latest mechanism for the phenomenon showed that wild-type p53 protein inhibit angiogenesis by thrombospondin[30]. The mRNA of thrombospondin-1 (TSP-1), which produced by fibroblasts, declined 14-17 fold after loss of wild-type p53. TSP-1 could inhibit angiogenesis by means of reducting plasminogen activator-inhibitor [16,18]. Concurrently, wild-type p53 could lower mRNA of VEGF, hinder induction of V-src to VEGF activity; on the other hand, mutant p53 induces the expression of the VEGF mRNA and potentiates 12-0-tetradecanoylphorbo1-13-acetate, which stimulates VEGF expression[33]. C-erbB-2 oncogene protein expression was associated with tumor histology grading stage, lymphatic metastasis and prognosis[34]. For the relationship between c-erbB-2 protein and angiogenesis, Toi et al and Horak et al concluded opposite results by the same method in breast carcinoma[31,35]. Our study showed that angiogenesis tend to increase along with the degree of c-erbB-2 protein expression increased in the period of breast atypical hyperplasia and breast carcinoma. But the difference was not significantly (P>0.05). The further study was necessary for the dispute of the relationship between c-erbB-2 and angiogenesis.

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