Multi-step process of human breast carcinogenesis: A role for BRCA1, BECN1, CCND1, PTEN and UVRAG

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Abstract. In the female population in Asia, systematic investigation concerning alterations in cancer-related genes in breast carcinoma is rare, and the correlation among oncogene or suppressor gene expression with tumor cell apoptosis, cell cycle regulation and tumor cell autophagy remains to be clarified. In this study, a tissue microarray consisting of 360 individual samples from three different breast tissues was generated. By comparing the expression of the tumor-suppressor genes (*BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG*) in ductal breast cancer and normal breast tissues, respectively, we were able to assign changes in the expression of these mRNAs to specific stages and allocate them to define the roles in the multi-step process of breast carcinogenesis. Tumor-suppressor genes, such as *BRCA1* and *BECN1*, usually had lower signals in the carcinomatous tissues (10.2 and 6.6%) compared to the normal tissues (31 and 32.6%), while stronger positive dots (positive cells >30%) usually existed in the normal tissues. The patients in the oldest age group had the lowest expression rate. Only *BECN1* and *CCND1* expression showed a significant association with patient age (p=0.030 and p=0.003). A significant association was observed between *BRCA1* and *BECN1* expression and tumor size (p=0.028 and p=0.021). *BECN1* gene expression was positively correlated with *UVRAG* and *PTEN* expression (p=0.006 and p=0.000). *CCND1* was negatively correlated with *PTEN*, *BECN1* and *BRCA1* expression (p=0.011, p=0.000 and p=0.000). Abnormal expression of *BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG* may play a role in human breast carcinogenesis through dysregulated mRNA expression. Overexpressed *CCND1* may shorten the G1 phase of the cell cycle, suppress cell apoptosis and contribute to the formation of invasive ductal carcinoma (IDC).

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Introduction

Breast cancer is the second most common type of cancer after lung cancer (10.4% of all cancer incidence, both genders counted) and the fifth most common cause of cancer-related mortality (1). In 2005, breast cancer caused 502,000 deaths worldwide (7% of cancer mortality; almost 1% of all mortality) (2). During recent years, the incidence rate of breast carcinoma has gradually increased in Asia and has become the second most common malignant tumor of Chinese women, with the number of new cases currently at approximately 60,000 in China annually (3). Ductal breast cancer is the most common type of breast cancer. Worldwide, 70-80 out of every 100 breast cancers diagnosed are of this type.

Breast cancer is considered to be the final outcome of multiple environmental and hereditary factors. Some of these factors include: i) lesions to DNA, such as genetic mutations (4); ii) failure of immune surveillance (5); iii) abnormal growth factor signaling in the interaction between stromal and epithelial cells facilitating malignant cell growth; iv) inherited defects in DNA repair genes, such as *BRCA1*, *BRCA2* and *p53*. Although numerous epidemiological risk factors have been identified, the cause of any individual's breast cancer is often unknown. In other words, epidemiological research provides information on the patterns of breast cancer incidence across certain populations, but not in a given individual (6).

Oncogenes act cooperatively with other genetic or epigenetic changes. In breast cancer, there has been much attention focused on oncogenic components of the cell signaling system, an example of which is *CCND1*. These are involved in transducing and modulating this signal, which results in a number of end events, including cell proliferation, alterations in drug sensitivity and DNA repair, angiogenesis, apoptosis and protease activity. The gene encoding *CCND1* is located on chromosome 11q13 and has been found to be overexpressed in 40-50% of invasive breast cancers and amplified in 10-20% of cases (7). When *CCND1* is complexed with its CDK partner,

the pRb tumor-suppressor protein is phosphorylated, releasing the transcriptional factor E2F and inducing proteins required for DNA synthesis. For example, breast cancer patients who have high levels of *CCND1* following mastectomy are at a higher risk for cancer recurrence than women with low levels of *CCND1* (8).

BRCA-1 codes for a protein of 1,863 amino acids, with a number of structural domains that hint at its function (9). A RING finger domain encodes a protein-binding domain at the amino terminus (10). BRCA-associated ring domain (BARD1) is a protein found to interact with BRCA-1 in the RING domain, and it may prove to have a tumor-suppressor function of its own. Two repeats in the carboxy terminus are similar to those observed in numerous DNA repair enzymes, including Rad9. Following genotoxic insult, BRCA-1 protein, along with BARD1 and Rad51, has been shown to localize to areas of damaged DNA, supporting a role in the regulation of transcription as well as in the repair of double-stranded DNA (11). *BRCA-1* protein with cyclin-dependent kinase and cyclin-A, D may also regulate the cell cycle.

The PTEN protein is a lipid phosphatase with putative tumor-suppressing abilities, including inhibition of the PI3K/Akt signaling pathway. Inactivating mutations or deletions of the PTEN gene, which result in hyper-activation of the PI3K/Akt signaling pathway, are increasingly being reported in human malignancies, including breast cancer, and have been related to features of poor prognosis and resistance to chemotherapy and hormone therapy (12). Inherited *PTEN* mutations, observed in Cowden syndrome, have been shown to increase the risk of breast and ovarian cancers (among others), although mutation of this gene in sporadic cases is uncommon (13,14).

The *BECN1* gene is localized to chromosome 17q21, a locus that is deleted in 50% of breast cancers (15). Recent studies have demonstrated that autophagy is related closely to the occurrence and development of tumors. Autophagic activity was found to be significantly reduced in beclin 1-deficient cells as assessed *in vivo* in mutant mice or *in vitro* in BECN1/embryonic stem (ES) cells. UV-irradiation resistance-associated gene (*UVRAG*), as a BECN1-interacting protein, associates with BECN1, enhances phosphatidylinositol-3-OH kinase class III [PI(3)KC3] activity and induces autophagosome formation. Expression of *UVRAG* may suppress the tumorigenicity and proliferation of human colon cancer cells, and it promotes autophagy in a manner that is interdependent of BECN1 (16). A number of studies have suggested that Bif-1 joins the UVRAG-BECN1 complex as a potential activator of autophagy and tumor suppressor (17,18).

Currently, all breast cancer cases should be tested for the expression, or a detectable effect, of the estrogen receptor (ER), progesterone receptor (PR) and HER2/neuproteins. These tests are usually carried out by immunohistochemistry, and a few involve *in situ* hybridization to test for oncogenes (*CCND1*) and tumor-suppressor genes (*BRCA1*, *BECN1*, *PTEN* and *UVRAG*). It is crucial to know whether the oncogene and suppressor gene RNA expression is different, comparing different steps in onset and progression of the disease. In addition, most studies of breast cancer-related genes have focused on gene expression and have reported less on the correlation between gene expression and cancer. In addition, studies on cancer-related genes have focused on Caucasian female patients. There are few reports associated with Asian females. Since the carcinogenesis of breast carcinoma may show discrepancies among various ethnicities (Caucasian female and Asian female), detailed information on gene amplification and abnormal expression of cancer-related genes in Chinese patients with breast carcinoma and its correlation with pathological parameters needs to be investigated.

Materials and methods

Tissue sample. The tissue samples used in this study were from patients who underwent surgery at the Department of General Surgery, Tangdu Hospital,The Fourth Military Medical University, Xi'an, China, from 2006 to 2009. All of the patients were Chinese and their mean age was 41.6 years.

Tissue microarray (TMA) construction. Tissue blocks of grossly apparent carcinoma and non-pathologic organs were trimmed to approximately 1.5x1.5x0.3 cm and were fixed immediately in 4% paraformaldehyde in phosphate-buffered saline (1% DEPC-PBS), pH 7.4, for 24 h. They were then dehydrated through an ethanol gradient and embedded in paraffin. Representative areas of the various lesions were carefully selected on H&E-stained sections and marked on individual paraffin blocks. The tissue cores were precisely arrayed into a new paraffin block using a TMA workstation (Beecher Instruments, Silver Spring, MD, USA). The final TMA consisted of 360 1-mm diameter TMA cores, each spaced at 0.8 mm from between core centers. An H&E-stained section was reviewed to confirm the presence of morphologically representative areas of the original lesions.

Preparation of digoxigenin-labelled probes for RNA in situ hybridization (IHS). cDNAs from 5 different human genes were cloned in order to synthesize ISH riboprobes. The cDNA was obtained from the total RNA of normal breast and breast cancer tissues by reverse transcription coupled with PCR by the use of primers (Table I). The cDNA was cloned into the pGEM-T (cat. #TM042) vector to obtain the pGEM-T/*BECN1* (333-992), pGEM-T/*BRCA1* (5046-5728), pGEM-T/*CCND1* (396-895), pGEM-T/*PTEN* (1414-1956) and pGEM-T/*UVRAG* (1323-2149) plasmids, which were used to synthesize riboprobes for RNA *in situ* hybridization assay.

The cDNA-containing plasmids were linearized, gel-isolated and used as templates for antisense and sense digoxigenin (DIG)-labeled riboprobe synthesis (Boehringer Mannheim, Mannheim, Germany). The transcription mixture (50 μl) included 1 μg of linearized template cDNA, ATP, GTP and CTP at 1 mM each, UTP 0.7 mM, DIG-UTP 0.3 mM, DTT 10 mM, RNase inhibitor (1 U/μl of transcription mix) and T3 or T7 RNA polymerase (1 U/μl of transcription mix). Transcription was performed for at least 2 h at 37˚C. The template cDNAs were then digested using RNase-free DNase (2 μl at 1 U/μl, 30 min at 37˚C), and all reactions were terminated by adjusting the reaction volume to 100 μl with Tris/EDTA (10/1 mM, pH 8.0). The riboprobes were then purified through two precipitation steps by addition of 100 μl NH_4-acetate 4 M and 500 μl EtOH 100%, and were centrifuged for 30 min at 4˚C in a microfuge. The pellet was resuspended in 200 μl DEPC-treated water. The synthesized

Table I. Primer sequences used for amplifying the studied genes.

Primer		Sequence 5'-3'	Product (bp)
BECN1	Sense	AGCCATTTATTGAAACTCCTCG	660
	Antisense	TATTGATTGTGCCAAACTGTCC	
BRCA1	Aense	GCTGCTCATACTACTGATACTGC	683
	Antisense	GCTACACTGTCCAACACCCACT	
CCND1	Sense	TGGATGCTGGAGGTCTGCGAGGAA	500
	Antisense	AGGCGGTAGTAGGACAGGAAGTTGTT	
PTEN	Sense	AGGGACGAACTGGTGTAATGAT	543
	Antisense	CACGCTCTATACTGCAAATGCT	
UVRAG	Sense	CTGTTGCCCTTGGTTATACTGC	827
	Antisense	GATGATTTCTTCTGCTTGCTCC	

riboprobe (5-10 μg) was obtained from 1 μg of cDNA matrix. The DIG-incorporation into the probes was controlled by dot spots. DIG was visualized with an anti-DIG antibody coupled to alkaline phosphatase.

RNA in situ hybridization. Deparaffinized sections mounted on Denhardt-coated glass slides were treated with pepsin (0.25 mg/ml in DEPC H_2O-HCl) for 25-30 min at 37˚C in a water bath. The treated sections were then processed for *in situ* hybridization at 41-48˚C for 16-24 h. The hybridization mixture contained the labeled oligonucleotide probe, 50% formamide, 10 mmol/l Tris-HCl, 1 mmol/l vanadyl-ribonucleoside complex (Sigma 94740, St. Louis, MO, USA), 1 mmol/l CTAB (Sigma 855820), pH 7.0, 0.15 mol/l NaCl, 1 mmol/l EDTA, pH 7.0, 1X Denhardt's mixture and 10% dextran sulfate. Hybridization was performed in a box saturated with a 5X SSC-50% formamide solution to avoid evaporation. Due to the different probes, a different pilot-experiment was applied to produce optimum results. Post-hybridization, slides were washed three times for 30 min in 0.1 mol/l TBS at room temperature, then treated with 1% blocking reagent (Boehringer Mannheim) in TBS (100 mmol/l Tris, pH 7.5, 150 mmol/l NaCl) with 0.03% Triton X-100 for 30 min at room temperature and incubated for 30 min with an anti-DIG alkaline phosphatase-conjugated antibody (Boehringer Mannheim; 11093274910) diluted 1:500 in TBS, 0.03% Triton X-100 and 1% blocking reagent. After washing three times for 15 min in TBS, 0.05% Tween, slides were rinsed in DAP-buffer (100 mmol/l Tris, 100 mmol/l NaCl and 50 mmol/l $MgCl_2$, pH 9.5) and, subsequently, hybridization signals were visualized using nitroblue tetrazolium and 5-brom-4-chlor-3-indolyl phosphate as substrates.

Statistical analysis. All cases were first grouped as positive or negative to calculate the percentages of positive and negative cases, as described. The χ^2 contingency test was used to evaluate the differences among groups. Analyses were performed using the statistical package SPSS 10.0 (SPSS, Chicago, IL, USA). $P<0.05$ was considered to denote statistical significance.

Results

Clinicopathological data. The clinical characteristics of the primary tumors are shown in Table II. A total of 105 patients were initially treated with breast conserving surgery, 125 by mastectomy and 11 by subcutaneous mastectomy. Information on adjuvant treatment was available for 97 cases. There were 250 invasive ductal carcinomas (IDCs), 2 lobular carcinomas and 1 mucinous carcinoma. In all breast carcinomas, tumors were moderately and poorly differentiated in 161 cases (64.4%) and 45 cases (18.0%). A total of 44 out of 250 cases (17.6%) were well-differentiated (Table II).

Table II. Histopathological characteristics of 250 cases of breast cancer.

Tumor type	n (%)
Invasive ductal carcinoma	250 (98.8)
Invasive lobular carcinoma	2 (0.8)
Mucinous carcinoma	1 (0.4)
Histological grading	
G1	44 (17.6)
G2	161 (64.4)
G3	45 (18.0)
Tumor extent	
pT1 (pT1a, pT1b, pT1c)	23 (9.20)
pT2	168 (67.2)
pT3	26 (10.4)
pT4 (pT4a, pT4b, pT4c, pT4d)	33 (13.2)
Lymph node status	
pN0	89 (35.6)
pN1	127 (50.8)
pN2	30 (12.0)
pNX	4 (1.6)
Metastases	
pM0	237 (94.8)
pM1	9 (3.6)
pMX	4 (1.6)

RNA in situ hybridization study. RNA *in situ* hybridization differences for *BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG* were found following categorization of the variables according

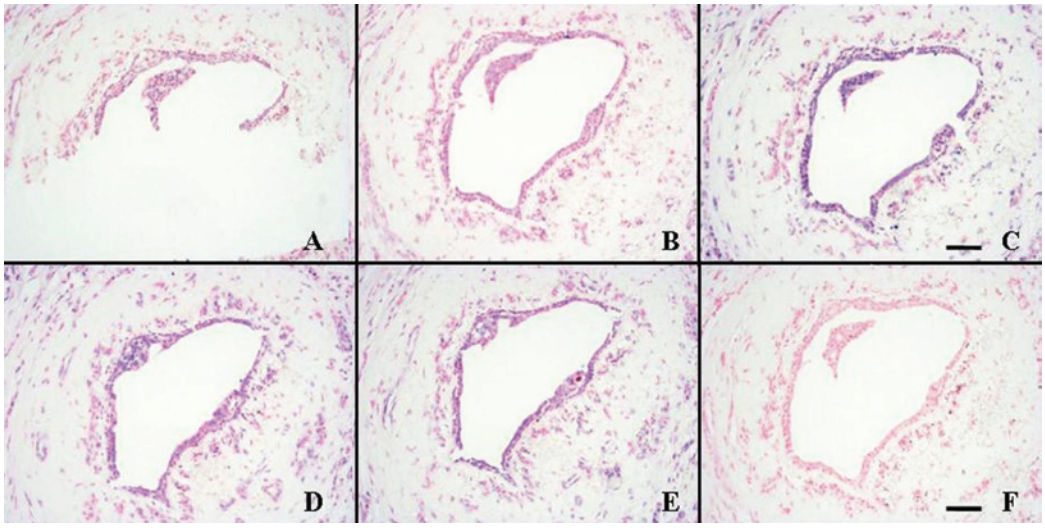

Figure 1. RNA *in situ* hybridization study in invasive ductal carcinoma and a negative control. RNA expression of (A) *BRCA1*, (B) *BECN1*, (C) *CCND1*, (D) *PTEN* and (E) *UVRAG*. (F) Negative control. Scale bars, 50 μm.

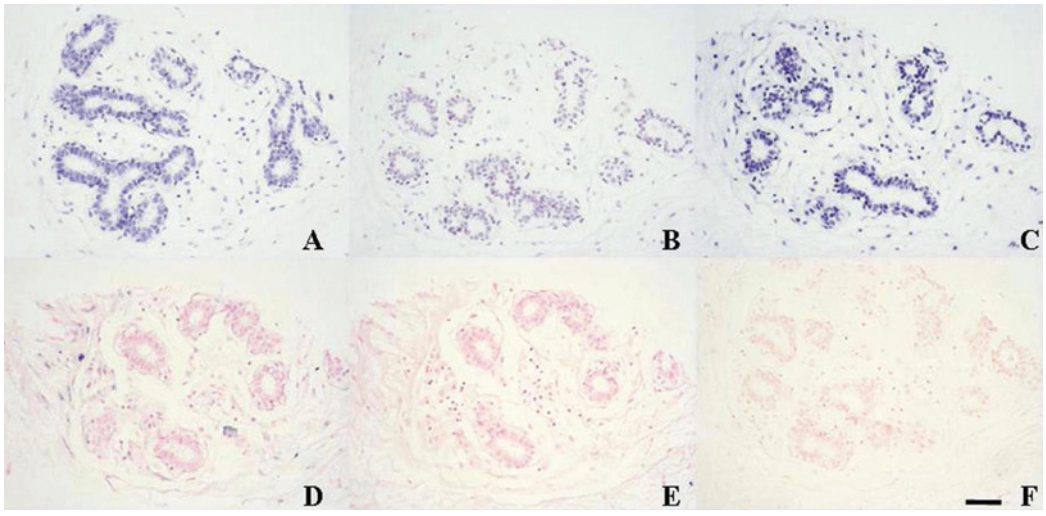

Figure 2. (A-E) RNA *in situ* hybridization study in normal and negative control. RNA expression of (A) *BRCA1*, (B) *BECN1*, (C) *CCND1*, (D) *PTEN* and (E) *UVRAG*. (F) Negative control. Scale bars, 50 μm.

to the thresholds defined previously between IDC and normal control tissue. The results were assigned intensity and percentage scores based upon the signal intensity of positive staining and the number of cells staining within the sample, respectively (Figs. 1 and 2). Typically, results of the R&ISH were observed as amethyst positive dots on arrays, located in the cytoplasm or nucleus. Tumor grading was classified according to the World Health Organization System. The presence of occasional tumor cells without detectable overexpression may be attributed to truncated cells that had lost genetic material during sectioning or tissue pre-treatment prior to hybridization.

mRNA expression of genes and its correlation with clinicopathological parameters in IDC. We analyzed significant differences in the *in situ* hybridization profile of mRNA expression of all genes according to histological grade. For example, positive expression of *BRCA1* was noted in all 250 cases of IDC and 96 cases of normal breast tissue, 35/44 cases (79.5%) of early-stage breast carcinoma, 107/161 cases (66.5%) with moderate tumor differentiation, 12/44 cases (26.7%) with poor tumor differentiation and 84/96 cases (87.5%) of normal breast tissue were defined as having positive expression (χ^2=2.083, p=0.000). Our study revealed that patients with poor tumor differentiation usually exhibited a lower oncogene expression rate, while patients with well-differentiated tumors always had a higher expression rate. For *BECN1*, 40/44 (90.9%) well-differentiated cases showed positive expression, but only 9/45 cases (20%) of poorly differentiated carcinoma exhibited *BECN1* expression. Tumor-suppressor genes, such as *BRCA1* and *BECN1*, usually had weaker signals in the carcinomatous tissues (10.2 and 6.6%) than those in the normal tissues (31 and 32.6%); stronger positive dots (positive cells >30%) usually existed in normal tissues (Table III). However, the oncogene *CCND1* had stronger signals in carcinomatous tissues (50.4%) compared to normal tissues (24.2%) (Fig. 3). Abnormal expression was usually involved in almost all of the tumor cells within the array element.

Table III. Correlation between mRNA expression of the cancer-related genes and clinicopathological parameters.

Item	*BRCA1*		*BECN1*		*CCND1*		*PTEN*		*UVRAG*	
	Positive (%)	P-value	Positive (%)	P-value	Positive (%)	P-value	Positive (%)	P-value	Positive (%)	P-value
Histological grading										
G1	35 (79.5)	0.000	40 (90.9)	0.000	43 (97.7)	0.000	34 (77.3)	0.000	16 (36.4)	0.000
G2	107 (66.5)		72 (44.7)		148 (91.9)		89 (55.3)		13 (8.10)	
G3	12 (26.7)		9 (20.0)		17 (37.8)		10 (22.2)		29 (64.4)	
Normal breast	84 (87.5)		73 (76.0)		75 (78.1)		52 (54.2)		37 (38.5)	
Age (years)										
<35	17 (73.9)	0.158	15 (65.2)	0.030	22 (95.7)	0.003	12 (52.2)	0.900	8 (34.8)	0.372
36-60	118 (62.4)		94 (49.7)		161 (85.2)		102 (54.0)		41 (21.7)	
>60	19 (50.0)		12 (31.6)		25 (65.8)		19 (50.0)		9 (23.7)	
Tumor extent										
pT1	14 (60.9)	0.028	11 (47.8)	0.021	21 (91.3)	0.112	14 (60.9)	0.630	6 (26.1)	0.054
pT2	94 (56.0)		74 (44.0)		133 (79.2)		85 (50.6)		41 (24.4)	
pT3	21 (80.8)		20 (76.9)		24 (92.3)		14 (53.8)		13 (50.0)	
pT4	25 (75.8)		16 (48.5)		30 (90.9)		20 (60.6)		8 (24.2)	
Lymph node status										
pN0	59 (66.3)	0.349	45 (50.6)	0.619	79 (88.8)	0.654	37 (41.6)	0.005	20 (22.5)	0.755
pN1	72 (56.7)		64 (50.4)		111 (87.4)		73 (57.5)		29 (22.8)	
pN2	19 (63.3)		18 (60.0)		28 (93.3)		22 (73.3)		5 (16.7)	

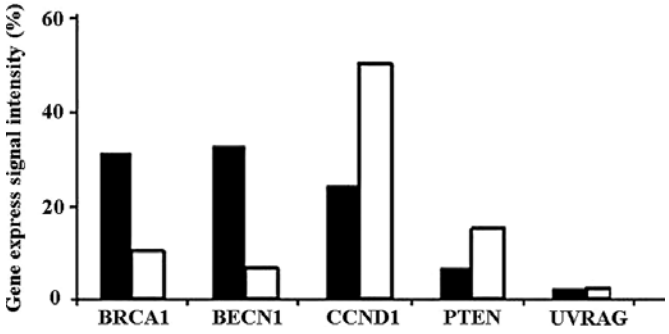

Figure 3. Signal intensity of the gene expression.

Table IV. Expression of *BRCA1*, *BECN1*, *PTEN* and its correlation with *CCND1* expression in IDC.

Variables	No.	*CCND1*				P-value
		-	+	++	+++	
BRCA1						
-	96	35	29	12	20	
+	74	3	7	27	37	
++	49	3	5	7	34	0.000
+++	31	1	6	4	20	
BECN1						
-	129	36	34	27	32	
+	63	5	6	12	40	
++	45	1	4	8	32	0.000
+++	13	0	3	3	7	
PTEN						
-	117	33	22	25	37	
+	55	4	6	2	43	
++	40	2	11	12	15	0.011
+++	38	3	10	9	16	

Patients were divided into three groups corresponding to age at diagnosis. Each group covered 25-35 years of age. Only *BECN1* and *CCND1* expression showed a significant association with patient age (p=0.030 and p=0.003). The patients in the oldest age group had the lowest expression rate.

No significant association was found between *CCND1* and *UVRAG* expression and tumor size and lymph node status. However, a significant association was observed between *BRCA1* and *BECN1* expression and tumor size (p=0.028 and p=0.021). The cases with a larger tumor size had a higher positive rate of *BRCA1* and *BECN1* expression. Tumors >5.0 cm showed a positive expression rate of 80.8% (21/26) for *BRCA1*, and it was significantly higher compared to the cases with tumors >2.0 cm, which showed an expression rate of 56% (94/168). There was a significant association between the *PTEN* mRNA expression and lymph node metastasis (p=0.005). A total of 37 out of 89 cases (41.6%) with no regional lymph node metastasis, 73/127 cases (57.5%) with metastasis to movable ipsilateral axillary lymph node, whereas 22/30 cases (73.3%) with metastasis to ipsilateral axillary lymph node showed expression of *PTEN* mRNA (Table III).

Expression of BRCA1, BECN1, PTEN and its correlation with CCND1 expression in IDC. Positive expression of *CCND1* was observed in almost all 250 carcinoma cases. A negative

Table V. Expression of *BECN1* and its correlation with *UVRAG* expression in IDC.

Variables	No.	*BECN1*				P-value
		-	+	++	+++	
UVRAG						
-	192	125	33	32	2	
+	43	4	26	11	2	
++	9	0	4	2	3	0.006
+++	6	0	0	0	6	

Table VI. Expression of *CCND1* and *BECN1* and its correlation with *PTEN* expression in IDC.

Variables	No.	*PTEN*				P-value
		-	+	++	+++	
CCND1						
-	42	33	4	2	3	
+	45	22	6	11	10	
++	48	25	2	12	9	0.011
+++	115	37	43	15	16	
BECN1						
-	129	81	24	11	13	
+	63	23	22	12	6	
++	45	8	7	15	15	0.000
+++	13	5	2	2	4	

correlation was observed between *BRCA1* and expression of *CCND1* (p=0.000). For *BRCA1*, 74 (29.6%) cases showed a weak positively stained cytoplasmic signal and were defined as + and 49 (19.6%) cases and 31 (12.4%) cases were defined as ++ and +++, respectively; however, for *CCND1* 47 (18.8%) cases exhibited a weak positively stained cytoplasmic signal and were defined as + and 50 (20.0%) cases and 111 (44.4%) cases were defined as ++ and +++, respectively. The cases with high *BRCA1* tended to have a lower probability of expressing *CCND1*. A total of 121 out of 250 cases (48.4%) demonstrated positive expression of *BECN1* and 133/250 cases (53.2%) demonstrated positive expression of *PTEN*. *BECN1* or *PTEN* showed a significantly negative correlation with *CCND1* expression (p=0.000 and p=0.011) in IDC (Table IV).

Expression of BECN1 and its correlation with UVRAG expression in IDC. *BECN1* binds *UVRAG* to target the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. A positive correlation was observed between *UVRAG* and expression of *BECN1* (p=0.006). For *BECN1*, 63 (25.2%) cases showed a weak positively-stained cytoplasmic signal and were defined as +, and 45 (18.0%) cases and 13 (5.2%) cases were defined as ++ and +++, respectively; in addition, for *UVRAG* 43 (17.2%) cases were found to have a weak positively stained cytoplasmic signal and were defined as +, while 9 (3.6%) cases and 6 (2.4%) cases were defined as ++ and +++, respectively. The cases with low *UVRAG* tended to have a lower probability of expressing *BECN1* in IDC (Table V).

Expression of CCND1 and BECN1, and its correlation with PTEN expression in IDC. We observed that the expression of *BECN1* had a positive correlation with the expression of *PTEN* (p=0.000). For *BECN1*, 63 (25.2%) cases showed a weak positively stained cytoplasmic signal and were defined as +, while 45 (18.0%) cases and 13 (5.2%) cases were defined as ++ and +++, respectively. In addition, for *PTEN* 55 (22.0%) cases exhibited a weak positively stained cytoplasmic signal and were defined as +, while 40 (16.0%) cases and 38 (15.2%) cases were defined as ++ and +++, respectively. The cases with high *BECN1* tended to have a higher probability of expressing *PTEN* in IDC (Table VI).

Discussion

TMA technology was used for our study as it allowed the analysis of a large number of samples and markers without producing methodological variations. Most previous reports on the expression of mRNA have utilized Northern blotting, dot blot or PCR-based approaches, while a few have involved *in situ* hybridization. Several normal tissues were dominated by adipose cells, differing greatly from tumor tissue in its epithelial cellularity. Normal and tumor tissues may not be rigorously compared by techniques involving RNA extraction from total tissue (19,20). Therefore, conclusions such as 'increased expression' may be more difficult to make from studies with Northern blotting, dot blot and PCR-based techniques that require RNA extraction from tissues that have not been fastidiously micro-dissected for the selection of tumor cells. All probes theoretically detect mRNA expression of wild-type and a great majority of mutant type genes, if transcriptionally active, since the oligonucleotide recognizes the DNA sequence near the 5' end in which mRNA transcription is considered to start. The data on *BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG* RNA *in situ* hybridization most commonly studied in associated tumors were similar to those reported previously (21-25). In addition, certain tissue positive rates were higher than previous reports, confirming the usefulness of the TMA approach.

In this study, all cases showed significant differences between gene expression and histological grade (p=0.000). The well-differentiated and normal breast tissue usually had a higher positive rate, while the poorly differentiated tissue always had a lower positive rate. For the expression of PTEN mRNA, 34/44 (77.3%) cases with well-differentiated tumors, and 52/96 (54.2%) normal controls showed positive signals, while the moderately and poorly differentiated breast carcinoma cases had a rate of PTEN expression of 55.3 and 22.2%, respectively. Tumor-suppressor genes, such as *BRCA1* and *BECN1*, usually had weaker signals (positive cells <30%) in carcinomatous tissues (10.2 and 6.6%) than those in the normal tissue (31 and 32.6%), while stronger positive dots (positive cells >30%) usually existed in normal tissue. By contrast, the oncogene *CCND1* had stronger signals in carcinomatous tissues (50.4%) compared to those in normal tissues (24.2%). We hypothesized that the abnormal expression of *BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG* may play a key role in

human breast carcinogenesis through regulating expression of its mRNA; overexpression of *CCND1* may contribute to the formation of IDC, however, normally expressed *BRCA1*, *BECN1* and *PTEN* in breast cancer may block the formation of IDC. The results of Steeg and Zhou (7) showed that *CCND1* has been found to be overexpressed in 40-50% of invasive breast cancers and amplified in 10-20% of cases. Expression of *CCND1* was found to play a significant role in the early staging of carcinogenesis in Caucasian females with breast carcinoma. We believe that the expression of *CCND1* plays the same role in Chinese patients with breast carcinoma.

Only *BECN1* and *CCND1* expression showed a significant association with patient age (p=0.030 and p=0.003). The patients in the oldest age group had the lowest expression rate. In addition, a significant association was observed between *BRCA1* and *BECN1* expression and tumor size (p=0.028 and p=0.021). The *PTEN* mRNA expression had a significant association with lymph node metastasis (p=0.005).

The cell cycle is crucial for the control of growth in cells and that is just what cancer is, a loss in the control of cell growth. The genes *BRCA1*, *BECN1*, *CCND1* and *PTEN* are involved in cell cycle regulation. The *CCND1* gene codes for a cell cycle protein which specifically acts during the G1 phase; upon interaction with cyclin-dependent kinases (CDK4 or CDK6) *CCND1* phosphorylates the p105-RB protein and thereby promotes progression in late G1, thus favoring entry into the S phase. Ectopic overexpression of *CCND1* has been shown to result in a shortened G1 phase and increased genetic instability, possibly due to a bypass of cell cycle checkpoints (26). *BRCA1* is involved in all phases of the cell cycle and regulates orderly events during cell cycle progression. BRCA1 deficiency consequently causes abnormalities in the S-phase checkpoint, the G_2/M checkpoint, and the spindle checkpoint and centrosome duplication (27). PTEN may induce a specific reduction in cyclin D3 levels and an associated increase in the amount of the inhibitor $p27^{KIP1}$ complexed with CDK2. Enforced expression of cyclin D3 was found to abrogate the PTEN-induced cell cycle arrest (28). *BECN1* inhibits the growth of cancer cells and decreases the expression levels of cyclin E and phosphorylated Rb. The present results showed that *BRCA1*, *PTEN* and *BECN1* had a significantly negative correlation with positive expression of *CCND1*. These cases had a higher *CCND1* mRNA expression and a lower suppressor gene expression was observed, which is thought to be an imbalance in the CCND1/CDK complex or to decrease the nuclear availability of *CCND1* through the Akt/PKB pathway.

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double or multimembrane autophagic vesicles, and their delivery to and subsequent degradation by the cell's own lysosomal system. A number of studies have raised the possibility that the breakdown of the autophagic process may contribute to the development of cancer. UVRAG is a BECN1-interacting protein. *UVRAG* association with *BECN1* enhances phosphatidylinositol-3-OH kinase class III [PI(3)KC3] activity and induces autophagosome formation. Our results showed that *BECN1* was markedly associated with positive expression of *UVRAG* (p=0.006). These results agree with the conclusion of other investigators. The results of Liang *et al* (17) showed that the UV irradiation resistance-associated gene is a positive regulator of the BECN1-PI(3)KC3 complex and may monoallelically mutate at a high frequency in human colon cancers, and associate with the BECN1-Bcl-2-PI(3)KC3 multiprotein complex, where *UVRAG* and *BECN1* interdependently induce autophagy (29,30). We hypothesized that the loss-balance expression of *UVRAG* and *BECN1* may affect normal cells in the process of autophagy which has an accelerating role in the breast carcinogenesis of Chinese women.

Apoptosis is the process of programmed cell death (PCD) and a frequent phenomenon in breast cancer. The apoptotic process is controlled by inducers and repressors, and the balance between these stimuli determines whether the cell cycle enters mitosis or apoptosis. PTEN and BECN1 induce cell apoptosis, but CCND1 shortens the G1 phase, increases genetic instability and bypasses cell cycle checkpoints. Our study revealed that the expression of PTEN was positively correlated to the expression of BECN1 (p=0.000), and had a negative correlation with the expression of CCND1 (p=0.011). PTEN-induced apoptosis is achieved mainly through its lipid phosphatase activity. PTEN through the PI3K/AKT signal transduction pathway from phosphoric acid reduces the level of AKT. AKT reduces the apoptosis factor and enhances the activity of anti-apoptotic protein. Excessive activation of AKT induces cells to lose their ability of apoptosis. By contrast, a PTEN-deficiency is likely to boost the Akt pathway, while the deficient cells show a significantly reduced sensitivity to agonist-induced apoptosis (31). We believe that the abnormal expression of the tumor-suppressor genes (*BECN1* and *PTEN*) and up-regulated oncogenes (*CCND1*) may disrupt apoptosis, leading to tumor initiation, progression or change in the promotion of apoptosis, thereby producing selective pressure to override apoptosis during multi-stage carcinogenesis. Finally, as a factor closely related to cancer, *CCND1* should open up new avenues for the study of the multi-step process of human breast carcinogenesis, since *CCND1*, known as an oncogene involved in the cell cycle, apoptosis and a number of other biological functions, is frequently up-regulated in numerous tumor types (32). It would be of utmost interest to determine whether *CCND1* is overexpressed in IDC and, if so, the overexpression of this oncogene may be considered as one of the most crucial early events in altered tumor tissues.

Cell cycle regulation, autophagy and apoptosis are closely linked to cancer. If genes that control cell cycle regulation, autophagy and apoptosis are overexpressed or expression is absent, normal breast cells may be altered into cancer cells. It would be meaningful to analyze lower grade tumors and premalignant lesions using the same measurement tools, to determine whether the oncogene and suppressor gene expression is different, comparing various steps in the onset and progression of the disease. Follow-up is currently being carried out to obtain information on 5-year survival rates of the patients and the protein level of those genes by immunohistochemical staining. Data will be provided in our subsequent study.

In conclusion, abnormal expression of *BRCA1*, *BECN1*, *CCND1*, *PTEN* and *UVRAG* may play a role in human breast carcinogenesis through dysregulated mRNA expression. Overexpressed *CCND1* may shorten the G1 phase, suppress cell apoptosis and contribute to the formation of IDC.

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