Covariation of copy number located at 16q22.1: New evidence in mammary ductal carcinoma

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Abstract. Copy number variation (CNV) is crucial for gene regulation in humans. A number of studies have revealed that CNV contributes to the initiation and progression of cancer. In this study, we analysed four breast cancer cell lines and six fresh frozen tissues from patients to evaluate the CNV present in the genome using microarray-based comparative genomic hybridization (aCGH). Six genes located at 16q22.1 were analysed by real-time PCR. The real-time PCR analysis revealed that the loss of CDH1/E2F4 may be associated with worse clinical and pathological findings. Interestingly, covariation of CDH1, CDH3, CTCF and E2F4 was found to be associated with triple negative breast cancer and HER-2 receptor status. In conclusion, our study supports the idea that CNV at 16q22.1 in breast cancer is a frequent event; furthermore, it reveals the covariation of CDH1, CDH3, CTCF and E2F4. The role of the covariation is more complex than a simple additive effect of these four separate genes, which may provide a novel target for breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer and is the leading cause of cancer deaths in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (1). As one of the most heterogeneous human tumours (2), multiple pathways are known to lead to tumourigenesis, and each pathway involves multiple steps contributing to tumourigenic progression. Chromosomal aberrations such as deletions, amplifications, or other forms of structural rearrangements have a major impact on tumour development (3).

Copy number variation (CNV), a significant source of genetic variation in humans, is one of substantial structural variations that affects the stability of the human genome (4,5). Constitutional CNVs might be associated with disease, including cancer predisposition (6). For example, changes in the copy number of genes such as ERBB2 (7) and c-MYC have been extensively documented in breast cancer and are present in model cell lines. Amplified (and overexpressed) genes are prime therapeutic targets; for example, the use of the drug trastuzumab against ERBB2 has been shown to improve breast cancer survival rates either alone or in combination with other treatments. This finding suggests that newly identified changes in copy number may provide new markers for breast cancer diagnosis, monitoring progression, therapeutic selection, and prognostic prediction.

The goal of our study was to find associations between CNVs and different tumour characteristics to identify new biomarkers for mammary ductal carcinoma.

Materials and methods

Specimens. Human breast tissues were collected from female breast cancer patients who were diagnosed and treated at the Third Affiliated Hospital of Harbin Medical University in 2010 (Table I). Primary tumour tissue and paired non-tumour tissues were initially frozen in liquid nitrogen within half an hour after surgery and then stored at -80˚C for long-term storage. All samples were inspected by pathologists to confirm that they consisted of >80% cancer cells. All non-tumour tissues were obtained from the same patient's breast tissue ≤5 cm away from the edge of tumour during mastectomy. Cell lines HCC70, HCC202, HCC1937 and HCC2218 were purchased from ATCC (Rockville, MD). The study was approved by the Research Ethics Committee of the hospital.

Experimental procedures

DNA preparation. Axygen® AxyPrep Multisource Genomic DNA Minprep kit (Axygen, Union City, CA, USA) and Pure Gene kit (Gentra Systems, Minneapolis, MN, USA) were used to extract genomic DNA as described by the manufacturer.

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Table I. Clinical and pathological composition of tumour patients.

	n	Per (%)
Total	101	100
Tumour size		
pT1	42	41.6
pT2	52	51.5
pT3	7	6.9
Node metastasis		
0	54	53.5
1-4	24	23.8
>5	23	22.7
TNM classification		
0	11	53.5
I	21	20.8
II	55	54.5
III	14	13.8
ER		
-	38	37.6
+ - +++	63	62.4
PR		
-	48	47.5
+ - +++	53	52.5
Her-2		
-	46	45.5
+ - +++	55	54.5
P53		
-	74	73.3
+ - +++	27	26.7
Ki67		
-	28	27.7
+ - +++	73	72.3
Family history		
Yes	89	88.1
No	12	11.9

The purity of the DNA was verified by the ratio A260/A280 = 1.80-2.0. The DNA was diluted to a concentration of 100 ng/μl, and aliquots stored at -80˚C.

aCGH. DNA from six fresh frozen tissue samples and four cell lines were investigated by microarray-based comparative genomic hybridization (aCGH) using the Agilent 244K oligonucleotide array according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA). Briefly, tumour tissue DNA was labelled with Fluoro Link Cy5-conjugated dUTP and non-tumour DNA was labelled with Fluoro Link Cy3-conjugated dUTP as the control. Then, 1.5 μg of gDNA was labelled and hybridised to the array at 65˚C for 40 h. For the cell lines, Agilent's 44k Human genome Gene Chip (Agilent Technologies, Palo Alto, CA, USA) was used to scan the whole genome. For the aCGH analysis, 100 ng of DNA was digested by Alu 1 and Rsa 1 (Invitrogen, Carlsbad, CA, USA) and labelled in the same manner. The hybridisation reaction was performed on an Agilent 2565AA DNA microarray scanner. Microarray images were analysed using Feature Extraction software. Finally, CGH Analytics software was used to analyse the data by the z-scoring method, with the parameters of a window of 1 M and threshold of 4.

Real-time PCR. The copy number of candidate genes, CDH1, CDH3, CDH5, E2F4, CTCF and TRF2, were quantified using the real-time PCR method. Briefly, total genomic DNA was extracted from 101 mammary ductal carcinoma tumour samples and their non-tumour tissue samples. Experiments were performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Fast-Start Universal SYBR Green Master (ROX) (2x) (Roche, Germany) according to the manufacturer's istructions. Primers for the candidate genes were designed based on the sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih/org) using Primer 5.0 software (Table II). Comparative Ct (threshold cycle) method was employed to quantify gene copy number. Candidate gene copy number $=2^{-\Delta\Delta Ct}=2^{-[(Ct\ candidate\ gene\ -\ Ct\ \beta\text{-actin})cancer\ group\ -\ (Ct\ candidate\ gene\ -\ Ct\ \beta\text{-actin})control\ group]}$. All samples were tested at least twice, and the median Ct values were established; $2^{-\Delta\Delta Ct}$ >2.0 was classified as an amplification and $2^{-\Delta\Delta Ct}$ <0.5 as a deletion (8).

Statistical analysis. The χ^2 test and rank-sum test were used to evaluate associations of the clinical and pathological parameters with gene CNV. For each gene, patients were divided into amplified/non-amplified groups (amplification vs. normal + deletion) and loss/non-loss groups (deletion vs. normal + amplification). All statistical calculations were performed using SPSS version 17.0 for Windows. Associations were calculated with Spearman's rho. Two-tailed P-values of <0.05 were considered statistically significant. Unsupervised hierarchical cluster analysis (Euclidean distance, average linkage analysis) was performed using cluster 3.0 software. The k-means algorithm was used to classify the covariation into an amplification group, normal group and deletion group.

Results and Discussion

Complex copy number variations were found at 16q22.1 by aCGH. CNVs exist in both cancers and normal human genomes (4,5). Genome-wide screening for CNVs in breast cancer will facilitate the identification of tumour suppressor genes (TSG) and oncogenes. In this study, we identified ~200 CNV segments including gains and losses. The size of CNV amplifications ranged from 20 kb to 245 Mb with an average gain of 40 Mb across an average of 80 separate CNVs events. The size of CNV deletions ranged from 35 kb to 138 Mb with an average loss of 25 Mb across an average of 91 separate CNVs events. Interesting variations were detected at 16q22.1: two of the six

Table II. Sequences of the primers for the six candidate genes at 16q22.1.

Genes	Primers	Primer sequences 5'→3'	Accession no.
β-actin	F	GTCACCAACTGGGACA	NT_007819.17
	R	CGCTTTACACCAGCCTCAT	
CDH1	F	CGTCACCGCTTCCCTTCTT	NT_010498.15
	R	CCACCTCCTCCGACCTCACTT	
CDH3	F	TTCCTCACTCGCTCCTCC	NT_010498.15
	R	CCGTTTGGCTTTCTTTCC	
CDH5	F	ATCCACCCGCCACAGTTT	NT_010498.15
	R	TCGGTGCTTGGTCTTCATCC	
CTCF	F	ACGCTTTCTATGTCTCCTT	NT_010498.15
	R	AACTATCAATCAACGCTACTG	
E2F4	F	GGAGCACCGCCTCACTAA	NT_010498.15
	R	ACACGCCAGGGAAGAGTT	
TRF2	F	GCTCTTCCCACTTTACCC	NT_010498.15
	R	GAATAGCCTTCACATCTTT	

F, forward; R, reverse.

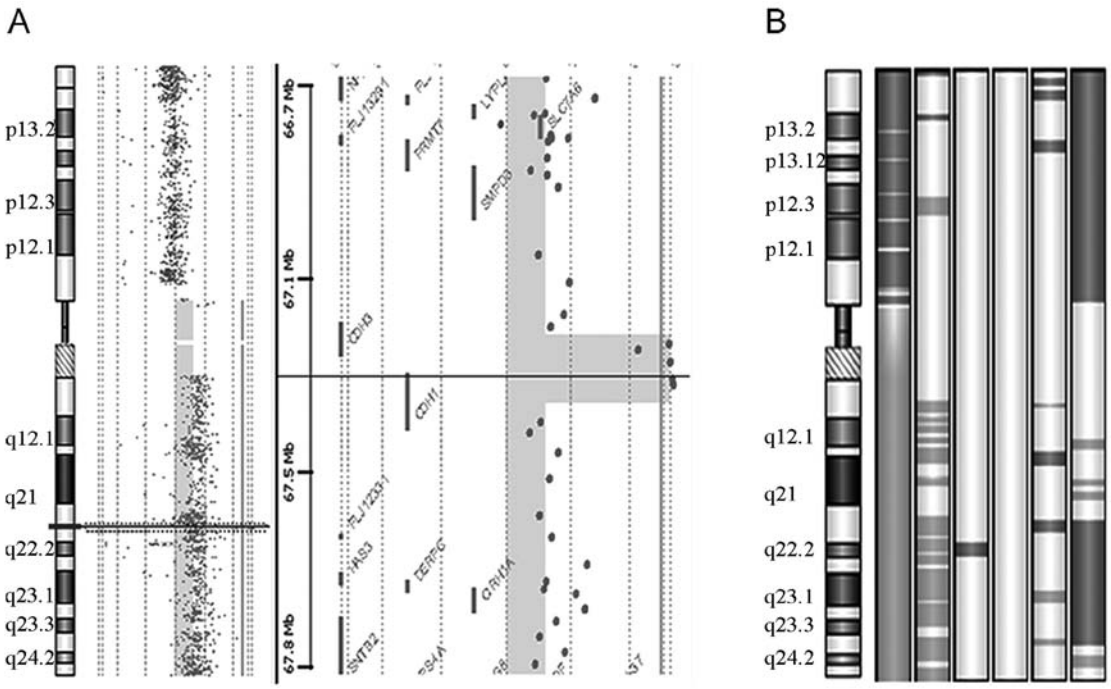

Figure 1. (A) A 30-fold copy number amplification was detected at 16q22.1 in breast cancer cell line HCC2218 by aCGH; (B) Deletion at 16q22.1 was detected in two of the six breast tumour tissues by aCGH. *Dark gray represents amplification and light gray represents deletion.

tissues had a deletion at this locus while one cell line had an amplification (Fig. 1). Although it has been reported that chromosome 16 is one of the most frequently altered chromosomes in breast cancer with a loss of heterozygosity at 16q occurring in about half of the low-grade ductal carcinomas and slightly more frequently in lobular carcinomas (9-13), the amplification of a DNA segment at 16q22.1 by >30-fold is described for the first time in the cell line HCC2218 by our study (Fig. 1). Thus, further research is needed to determine the significance of this distinct variation in a breast cancer.

Quantitation of candidate gene CNVs in 101 mammary ductal carcinoma patients by real-time PCR. Some candidate tumour suppressor genes are located in this region, such as CDH1

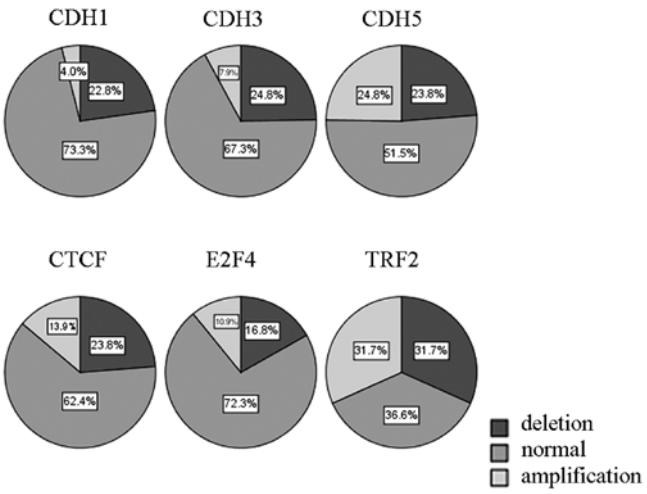

Figure 2. A pie chart of gray scale describing CNVs of CDH1, CDH3, CDH5, CTCF, E2F4, TRF2 in 101 breast cancer patients detected by real-time PCR method.

(14-18), CDH3 (19,20), CDH5 (21), E2F-4 (22), CTCF (23) and TRF2 (24,25). We selected these six genes for copy number quantitation in patients.

Sequence loss was commonly observed for all of the six candidate genes (Fig. 2). Among the 101 specimens, 22.8% showed a loss of CDH1 while the percentage showing amplification was only 4.0%. A similar finding was noted among CDH3, CTCF and E2F4 with losses in 24.8, 23.8 and 16.8% of the specimens, respectively, and with a lower corresponding percentage showing amplifications.

For CDH5 and TRF2, a total of 24.8 and 31.7% of the specimens had amplifications of CDH5 and TRF2, respectively, which is higher than that observed in the other four genes. Among these, 10.9% specimens showed a high number of TRF2 amplifications ($2^{-\Delta\Delta Ct}$ >10).

Unlike the findings in the HCC2218 cell line, patients tended to present with a loss at 16q22.1 in general. HCC2218 is a poorly differentiated cell line that is derived from the primary tumour of an invasive ductal carcinoma in an individual with a posi-

Table III. Association of CNVs with clinical and pathological features.

	TNM 0/1/2/3	Node 0/1/2	ER -/+	PR -/+	HER-2 -/+	Ki67 -/+	P53 -/+	Triple negative Yes/no	Family history Yes/no	Pathological classification 0/1/2/3
CDH1 loss/non-loss	P=0.009	P=0.022	P=0.717	P=0.621	P=0.958	P=0.055	P=0.538	P=0.204	P=0.845	P=0.747
CDH3 loss/non-loss	P=0.509	P=0.444	P=0.234	P=0.355	P=0.560	P=0.972	P=0.722	P=0.034	P=0.148	P=0.509
CDH5 loss/non-loss	P=0.431	P=0.304	P=0.132	P=0.034	P=0.273	P=0.081	P=0.455	P=0.181	P=0.407	P=0.100
CDH5 amp/non-amp	P=0.033	P=0.304	P=0.234	P=0.166	P=0.414	P=0.632	P=0.722	P=0.031	P=0.489	P=0.062
CTCF loss/non-loss	P=0.008	P=0.038	P=0.589	P=0.238	P=0.88	P=0.482	P=0.071	P=0.039	P=0.121	P=0.177
CTCF amp/non-amp	P=0.269	P=0.901	P=0.201	P=0.100	P=0.805	P=0.939	P=0.413	P=0.234	P=0.234	P=0.059
E2F4 loss/non-loss	P=0.039	P=0.758	P=0.767	P=0.536	P=0.711	P=0.865	P=0.784	P=0.402	P=0.987	P=0.641
TRF2 loss/non-loss	P=0.072	P=0.162	P=0.066	P=0.021	P=0.232	P=0.014	P=0.217	P=0.234	P=0.035	P=0.024

For significant associations, the corresponding P-values following χ^2 test are depicted in the table. For genes, loss/non-loss refers to deletion vs. normal + amplification while amp/non-amp refers to amplification vs. normal + deletion. For clinical and pathological features, 0/1/2/3 staging of TNM are followed for standard of TNM-UICC classification; nodal 0/1/2 refers to no lymph node metastasis, 1-3 lymph nodes metastasis and >4 lymph nodes metastasis, respectively; pathological classification 0, 1, 2 and 3 refers to ductal carcinoma *in situ*, infiltrating duct carcinoma I, II and III respectively; -/+ refers to negative and positive (ranging from + to +++), respectively.

Table IV. Relationship of candidate genes CNVs in 101 breast cancers.

	CDH1	CDH3	CDH5	CTCF	E2F4	TRF2
CDH1						
Coefficient correlation		0.645	0.567	0.477	0.618	0.592
Significance (2-tailed)		<0.001	<0.001	<0.001	<0.001	<0.001
CDH3						
Coefficient correlation			0.585	0.525	0.604	0.477
Significance (2-tailed)			<0.001	<0.001	<0.001	<0.001
CDH5						
Coefficient correlation				0.445	0.499	0.569
Significance (2-tailed)				<0.001	<0.001	<0.001
CTCF						
Coefficient correlation					0.496	0.505
Significance (2-tailed)					<0.001	<0.001
E2F4						
Coefficient correlation						0.482
Significance (2-tailed)						<0.001
TRF2						
Coefficient correlation						
Significance (2-tailed)						

tive family history of cancer and with lymph nodes metastases, which were highly positive for the expression of HER2, positive for the expression of p53, positive for the epithelial cell-specific marker epithelial glycoprotein-2 (EGP2) and for cytokeratin-19, but negative for the expression of the estrogen receptor (ER). However, cell lines do not always represent the genotypes of parental tumour tissues. Established cell lines carry cell lines-specific CNVs together with the aberrations detected in primary tumour tissues (26), and this might provide an explanation as to why no significant amplifications were detected in our cohort.

Association of single gene CNVs with clinical and pathological features. Associations between CNVs of these six genes with common clinical and pathological factors (age, histological type and grade, HER2, ER, PR, P53, Ki67, family history, tumour size) were analysed to obtain a more complete understanding of CNVs. A subset of the statistical results is shown in Table III.

Cadherins are calcium-dependent adhesion molecules that have been implicated in numerous cellular functions, ranging from controlling morphogenesis to suppressing tumour invasion and metastasis (27-29). CDH1 encodes E-cadherin, which is a calcium-dependent trans-membrane glycoprotein. It is involved in pathological cellular systems, such as the epithelial-mesenchymal transition, which is a process frequently related to tumour de-differentiation and infiltration metastasis. In our study, a significant association could be noted between the loss of CDH1 and worse clinical findings, such as a later TNM classification (P=0.009) and a greater frequency of lymph node metastases (P=0.022), supporting CDH1 as a TSG in breast ductal cancer. CDH3 encodes P-cadherin, P-cadherin expression shows a strong association with high histologic tumour grade scores, increased proliferation, Her-2 and p53 expression, a lack of the estrogen receptor and poor patient survival (19). In this study, no significant association was observed between CNVs of CDH3 and these aspects. VE-cadherin, encoded by CDH5, is up-regulated in invasive breast cancers, contributing to neovascularisation in tumours (30). However, the endothelial-specific VE-cadherin is low or absent in angiosarcomas, supporting a suppressive role for this protein in tumour progression (31). Amplification of CDH5 could be found in more triple negative breast cancers [ER(-), PR(-), HER-2(-)] than in non-triple negative breast cancer patients in this study (P=0.031), suggesting that more research is needed to further elucidate the role of CDH5 in breast cancer.

CTCF and E2F4 are two important transcription factors at this locus. CTCF is widely expressed in different aspects of gene regulation including promoter activation (32) and repression (33), hormone-responsive gene silencing (34), methylation-dependent chromatin insulation and genomic imprinting (35,36). In addition, CTCF can inhibit cell growth and induce cell cycle arrest at multiple stages (37). In our study, the loss of CTCF may result in a worse TNM classification (P=0.008) and an increase in lymph node metastases (P=0.038), but fewer triple negative breast cancer patients were found with a loss of CTCF, compared with non-triple negative patients (P=0.039). E2F4, another transcription factor, has been thought to be a tumour suppressor in breast cancer (22). The key roles of

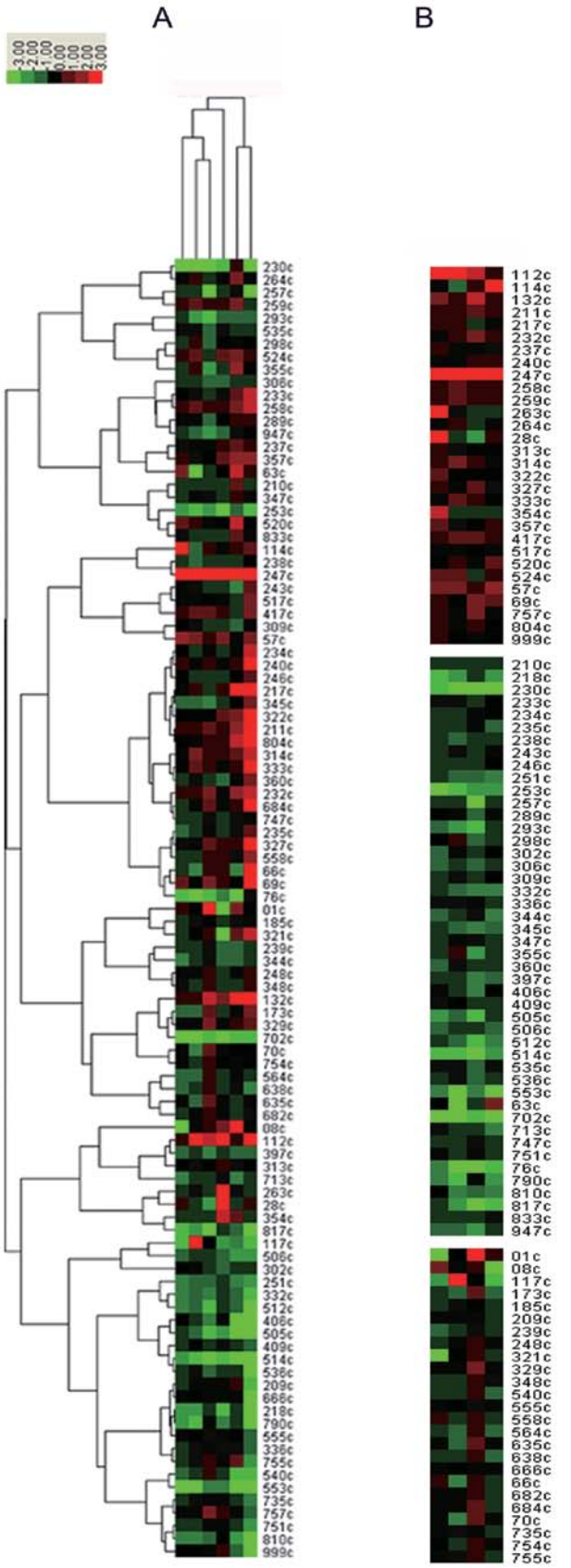

Figure 3. (A) Hierarchical cluster analysis of 101 breast cancer patients (vertical axis) analyzed by real-time PCR for 6 candidate genes (horizontal axis). DNA copy number gains and losses indicate as the color-bar; (B) Clustering of 101 mammary ductal carcinoma patients (vertical axis) by the k-means algorithm. The 4 genes (horizontal axis) used for the cluster analysis (left to right, E2F4 CDH3 CTCF CDH1). Depending on the situation of covariance, the samples were divided into 3 groups, the top group represents amplification, the bottom group represents loss.

E2F4 in preventing carcinogenesis are controlling the cell cycle, cell growth and apoptosis; moreover, E2F4 is associated with p130, p107 and pRb and is abundant in non-cycling cells (38). In our study, the loss of E2F4 was associated with a worse TNM classification (P=0.039), supporting E2F4 as a TSG in breast cancer.

The TRF2 gene encodes a ubiquitous protein that is related to telomerase activity. It maintains telomere structure and function (39). Additionally, the inhibition of TRF2 has been found to induce apoptosis (40) and has been implicated in some cancers, such as gastric cancer and leukaemia (24,41). We found that, in a considerable proportion of the patients, the amplification of TRF2 could be detected and that the loss of TRF2 might be related to a lower Ki67 positive ratio (P=0.012) and a higher PR positive ratio (P=0.012).

Covariation of CDH1, CDH3, CTCF and E2F4 was found as well as associations between the variation and clinical and pathological features. In cancers, chromosomal variation of not only one single specific gene but also of several linked genes often contributes to pathogenesis (42,43). Associations between CNVs of each gene were noted in our study (Table IV). Through hierarchical cluster analysis, an apparent cluster of CDH1, CDH3, CTCF and E2F4 was revealed, while CDH5 and TRF2 formed the other cluster (Fig. 3). This finding suggested that the genes CDH1, CDH3, CTCF and E2F4 are closely related to one another, so all specimens were divided into three groups (amplification/normal/deletion) using a covariation model of these four genes by the k-means algorithm. A total of 46 of the 101 patients (45.5%) showed deletions in this region while 30 of the 101 patients (29.7%) showed amplifications (Fig. 3). In this study, a covariation of CDH1, CDH3, CTCF and E2F4 was found for the first time. It is interesting that the other two genes, CDH5 and TRF2, map to the two ends of the locus while the other four genes are located in the middle (http://www.ncbi.nlm.nih/org). Using a χ^2 test on the clinical and pathological features, we surprisingly found that triple negative breast cancers more frequently belonged to the non-deletion group (normal group/amplification group) rather than the deletion group (P=0.032). These results suggest that the role of covariation is more complex than a simple additive effect and may provide novel insight for us to evaluate the disease.

In conclusion, our study supports that sequence loss at 16q22.1 occurs commonly in mammary ductal carcinoma and covariation of copy number, including CDH1, CDH3, CTCF and E2F4, were found for the first time. The role of covariation is more complex than a simple additive effect, which may provide novel insight into the evaluation of the clinical and pathological attributes of this disease. CDH1 and E2F4 are supported as important TSGs of mammary ductal carcinoma. CNV of CDH3, CDH5 CTCF and TRF2 was also found to play a role in breast cancer, but more evidence is needed to clarify the effects of these genes.

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