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Abnormal expression of Nek2 and β-catenin in breast carcinoma: clinicopathological correlations

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Aims: NIMA-related kinase 2 (Nek2) and β-catenin are important centrosome regulatory factors. The aim of this study was to detect the possible disparity in their expression among normal breast tissue, invasive ductal carcinoma (IDC), concomitant ductal carcinoma *in situ* (DCIS), and pure DCIS, and to explore its correlation with clinicopathological factors.

Methods and results: We used immunohistochemistry to detect protein expression of Nek2 and β-catenin in breast cancer tissues from 60 cases of pure DCIS, 348 cases of IDC and 137 cases of concomitant DCIS with that in normal breast tissues from the same 137 concomitant DCIS patients as controls. As compared with normal tissue, expression of Nek2 and β-catenin in the cytoplasm was significantly increased in IDC and DCIS ($P < 0.05$), and variation in expression was also observed in different grades of IDC ($P < 0.01$). Also, cytoplasmic expression of Nek2 and and of β-catenin were correlated with each other in IDC and DCIS ($P < 0.01$). In addition, they were both related to Ki67 immunoreactivity ($P < 0.05$). Furthermore, our study also revealed a correlation between their expression and some clinicopathological factors. We found that Nek2 cytoplasmic expression was associated with grade and tumour size ($P < 0.01$) in IDC, whereas β-catenin cytomembrane expression showed significant variation with grades, TNM stages, lymphoid node status, oestrogen receptor status, and molecular subtype ($P < 0.05$); a difference in expression was also observed between IDC and DCIS ($P < 0.05$). Also, β-catenin cytoplasmic expression was associated with TNM stage ($P < 0.05$). Expression of *Nek2* at the mRNA level was detected in 50 pairs of breast cancer specimens and matched normal tissues by reverse transcriptase polymerase chain reaction, and the result showed increased expression in IDC.

Conclusions: This study suggests that abnormal expression of Nek2 and β-catenin might be one of the mechanisms of tumorigenesis, especially of abnormal tumour proliferation. They may represent new potential targets for therapeutic intervention.

Keywords: breast invasive ductal carcinoma, ductal carcinoma *in situ*, Ki67, Nek2, β-catenin

Abbreviations: ASCO, American Society of Clinical Oncology; CISH, chromogenic *in-situ* hybridization; CK, cytokeratin; DAB, 3,3′-diaminobenzidine tetra-hydrochloride; DCIS, ductal carcinoma *in situ*; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; IHC, immunohistochemical; IRS,

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immunoreactivity score; Nek2, NIMA-related kinase 2; NLS, nuclear localization sequence; MT, microtubule; PBS, phosphate-buffered saline; PR, progesterone receptor; RT-PCR, reverse transcriptase polymerase chain reaction; siRNA, small interfering RNA

Introduction

NIMA-related kinase (Nek2) is a serine/threonine kinase located at the centrosome. It regulates centrosome cohesion and separation through phosphorylation of structural components of the centrosome. Nek2 exists in three forms, which are Nek2A, Nek2B and Nek2C in mammalian cells.[1] It is known that the aberrant regulation of Nek2 activity can lead to aneuploid defects of cancer cells.

A previous study[2] showed that β-catenin was located and functioned at the centrosome, which represents a new subcellular location. As a component of the centrosome linker, β-catenin works as a key regulator of centrosome separation in mitosis. Huang *et al.* [3] demonstrated a novel role of phosphor-β-catenin in microtubule (MT) organization at the centrosomes. Bahmanyar *et al.*[2] showed that β-catenin was one of the Nek2 substrates involved in centrosome separation. Centrosomes are held together by a dynamic linker regulated by Nek2 and its substrates, such as Rootletin and centrosomal Nek2-associated protein 1. β-Catenin binds to and is phosphorylated by Nek2 in a complex with Rootletin. When Nek2 activity is abnormally increased, it can disrupt the interaction of Rootletin with centrosomes, and result in binding of β-catenin to Rootletin-independent sites on centrosomes, which is an event that can make the centrosome separate abnormally.

It is known that centrosomes establishe the poles of the bipolar mitotic spindle, which controls the equal division of chromosomes. Supernumerary centrosomes[4] can create a mutator phenotype that promotes cancerous transformation. Ganem *et al.*[5] showed that extra centrosomes alone are sufficient to promote chromosome missegregation during bipolar cell division; this is consistent with the results of our former study[6] demonstrating that centrosomal aberrations may play key roles in the early stage of breast tumorigenesis. The malignant transformation is probably attributable to the overexpression of both mRNA and protein in the centrosome, resulting from amplification of centrosomal DNA.

Most of previous studies on Nek2 were conducted in cell lines; there have been few studies in breast tissue, and the relationship of Nek2 and β-catenin has also been rarely investigated in breast tissue. We detected their expression in different grades of breast invasive ductal carcinoma (IDC), concomitant ductal carcinoma *in situ* (DCIS), pure DCIS, and normal tissue. This research can provide some information on Nek2 and β-catenin expression in patient tissues, and may contribute to finding new targets for therapeutic intervention. In this study, we examined immunohistochemically the protein expression of Nek2 and β-catenin in 60 cases of pure DCIS, 348 cases of IDC, and 137 cases of concomitant DCIS, together with normal breast tissues from the same 137 patients as controls. mRNA expression of Nek2 was detected in 50 pairs of breast cancer specimens and matched normal tissues by reverse transcriptase polymerase chain reaction (RT-PCR).

Materials and methods

CASE SELECTION AND CLINICAL INFORMATION

For immunohistochemistry, 60 cases of pure DCIS, 348 cases of formalin-fixed and paraffin-embedded surgical samples of breast IDC and 137 cases of concomitant DCIS were collected randomly from a total of 5360 cases of IDC diagnosed from January 2007 to December 2009 from the archives of the Department of Breast Cancer Pathology and Research Laboratory Cancer Hospital of Tianjin Medical University (Tianjin, China). The normal tissues adjacent to tumours were collected from the same 137 patients (who underwent mastectomy). The diagnosis and histopathology results were reviewed independently by three pathologists (S.W., Y.N., and S.L.), using the World Health Organization criteria.[7] All patients were women, ranging in age from 28 to 83 years (mean 52 years); other clinicopathological information is shown in Table 1. None had received preoperative radiation or chemotherapy.

For RT-PCR, all breast cancer tissues and matched normal specimens were obtained from patients who underwent surgical resection from January 2007 to December 2009. After the standard procedure for clinical diagnosis was completed, additional tissues were cut into small pieces, snap frozen in liquid nitrogen, and stored in a −80°C freezer until use. This study was approved by the Ethics Committee of the Tumour Hospital of Tianjin Medical University.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed by using the labelled streptavidin–biotin technique, with antibodies

Table 1. Clinical information for invasive ductal carcinoma (IDC), concomitant ductal carcinoma *in situ* (DCIS), and pure DCIS

	IDC *n* = 348 *n* (%)	Concomitant DCIS *n* = 137 *n* (%)	Pure DCIS *n* = 60 *n* (%)
Age (years)			
≤50	150 (43.10)	65 (47.45)	27 (45.00)
>50	198 (56.89)	72 (52.55)	33 (55.00)
Histological grade			
I	70 (20.11)	45 (32.85)	16 (26.67)
II	166 (47.70)	68 (49.64)	29 (48.33)
III	112 (32.18)	24 (17.52)	15 (25.00)
TNM stage			
0			60 (100)
I	50 (14.37)	27 (19.71)	0 (0)
II	169 (48.56)	68 (49.64)	0 (0)
III	129 (37.07)	42 (30.66)	0 (0)
Tumour size (mm)			
≤20	79 (22.70)	44 (32.12)	14 (23.33)
20–50	242 (69.54)	84 (61.31)	41 (68.33)
>50	27 (7.76)	9 (6.57)	5 (8.33)
Lymph node status			
+	150 (43.10)	74 (54.01)	0 (0)
–	198 (56.90)	63 (45.99)	60 (100)
ER			
+	214 (61.49)	83 (60.58)	36 (60.00)
–	134 (38.51)	54 (39.42)	24 (40.00)
PR			
+	200 (57.47)	79 (57.66)	34 (56.67)
–	148 (42.53)	58 (42.34)	26 (43.33)
HER2			
+	79 (22.70)	39 (28.47)	17 (28.33)
–	269 (77.30)	98 (71.53)	43 (71.67)
Molecular subtype			
Luminal A	207 (59.48)	79 (57.66)	34 (56.67)
Luminal B	44 (12.64)	20 (14.60)	9 (15.00)
HER-2/*neu*	35 (10.06)	19 (13.87)	8 (13.33)
Basal-like	48 (13.80)	14 (10.22)	7 (11.67)
Unclassified	14 (4.02)	5 (3.65)	2 (3.33)

ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

against Nek2, β-catenin, Ki67, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) in all cases, and cytokeratin (CK)5/6 and epidermal growth factor receptor (EGFR) only in ER–/PR–/HER2– cases. Briefly, tissue sections were deparaffinized and rehydrated with xylene and a series of grades of alcohol. Antigen retrieval was carried out in 5 mM citrate buffer (pH 6.0) or EDTA (pH 9.0) for 2 min in an autoclave, and this was followed by cooling at room temperature for 45 min. After inactivation of endogenous peroxidase with 3% H_2O_2, sections were blocked with 10% normal goat serum for 30 min and then incubated with either anti-Nek2 antibody (1:100 dilution; Abcam, Cambridge, UK), anti-β-catenin antibody (1:50; Zymed, South San Francisco, CA, USA) or anti-Ki67 antibody (1:75; Zymed) at 4°C overnight, or with either anti-ER antibody (1:150; Zymed), anti-PR antibody (1:150; Zymed), anti-HER-2/*neu* antibody (1:50; Zymed), anti-CK5/6 antibody (1:100; Zymed) or anti-EGFR antibody (1:100; Zymed) at 37°C for 2 h. Normal mouse serum served as a negative control. After incubation with biotin-conjugated secondary antibody for 20 min at 37°C, and streptavidin–horseradish peroxidase (Zymed) for 20 min at 37°C, colour was developed by incubation with 3,3′-diaminobenzidine tetra-hydrochloride (DAB). The sections were counterstained with haematoxylin. All steps were preceded by rinsing with phosphate-buffered saline (PBS; pH 7.6).

Nek2 was expressed in the cytoplasm and/or nucleus and β-catenin was expressed in the cytomembrane and/or cytoplasm in tumour cells. Five areas of every slide were chosen randomly, and we counted 150–200 carcinoma cells per area; protein expression was assessed according to the immunoreactivity score (IRS).[8] The IRS is the product of immunoreactivity intensity (0, no reactivity; 1, weak reactivity; 2, moderate reactivity; 3, trong reactivity) and percentage of positive cells (0, 0% of cells reactive; 1, <25% of cells reactive; 2, 25–49% of cells reactive; 3, 50–74% of cells reactive; 4, ≥75% of cells reactive). The score of every slide is the average value of the five areas. Each case was graded by the product of the two scores as 0–1 (–, negative), 2–4 (+), 5–8 (++), and above 9 (+++), and +, ++ and +++ were defined as positive. Ki67 immunoreactivity was evaluated by the percentage of tumour cells with nuclear reactivity counted across five representative fields (–, <10% of cells reactive; +, 10–25% of cells reactive; ++, 26–49% of cells reactive; +++, >50% of cells reactive). According to guidelines[9] published by the American Society of Clinical Oncology (ASCO), ER/PR negativity by immunohistochemical (IHC) analysis is defined as <1% immunoreactive tumour cells. HER2 scores of 0 and 1 were considered to indicate negativity, and tumours were considered to be positive if the IHC score was 3, or if it was 2 and the tumours were chromogenic *in-situ* hybridization (CISH)-positive.[10] Cases were scored positive for CK5/6 and EGFR if cytoplasm and/or cytomembrane immunoreactivity was present in tumour cells (Figure 1C,D). Breast carcinoma were classified into five molecular subtypes,[11,12] including: luminal A (ER+ and/or PR+, HER2–), luminal B (ER+ and/or PR+, HER2+), basal cell-like (ER–, PR–, HER2–, CK5/6+ and/or EGFR+), unclassified (ER–, PR–, HER2–, CK5/6–, EGFR–), and HER-2/*neu* (ER–, PR–, HER2+).

CISH

The cases IHC scored as 2+ for HER2 were definitively assessed with CISH. Sections (4 mm in thickness) were incubated in SPOT-Light Heat Pretreatment Solution (Zymed) at 98–100°C for 15 min, washed with PBS, and digested with SPOT-Light Tissue Pretreatment Enzyme (Zymed) for 5 min. They were then washed with PBS and dehydrated through a graded series of ethanol. HER2 probe (Zymed) was applied to the slides, which were then coverslipped, sealed with rubber cement, and placed on a preprogrammed HYBrite (Vysis, San Diego, CA, USA) with the following settings: denaturation at 90°C for 5 min, and hybridization at 37°C for 16 h. The rubber cement was then removed, and the slides were immersed twice in SSC for 5 min, the first time at room temperature, and the second time at 70°C. They were then immersed in absolute methanol with 3% H_2O_2 for 10 min, and incubated with CAS-Block (Zymed) for 20 min. Hybridization signals were detected after sequential incubations with mouse anti-digoxigenin (Zymed), polymerized horseradish peroxidase anti-mouse antibody, and DAB, and counterstained with Mayer's haematoxylin (Zymed) for 3 min. CISH results were evaluated according to the criteria of the ASCO/College of American Pathologists guidelines.[9] A positive HER2 result was considered to be more than six *HER2* gene copies per nucleus (Figure 1A) and a negative result fewer than 4.0 *HER2* gene copies per nucleus (Figure 1B) in 50% of the tumour cells.

SEMIQUANTITATIVE RT-PCR

Nek2 mRNA expression was detected in 50 pairs of primary breast cancer and matched normal breast tissues by semiquantitative RT-PCR with TRI Reagent (Sigma, Saint Louis, MO, USA), according to the

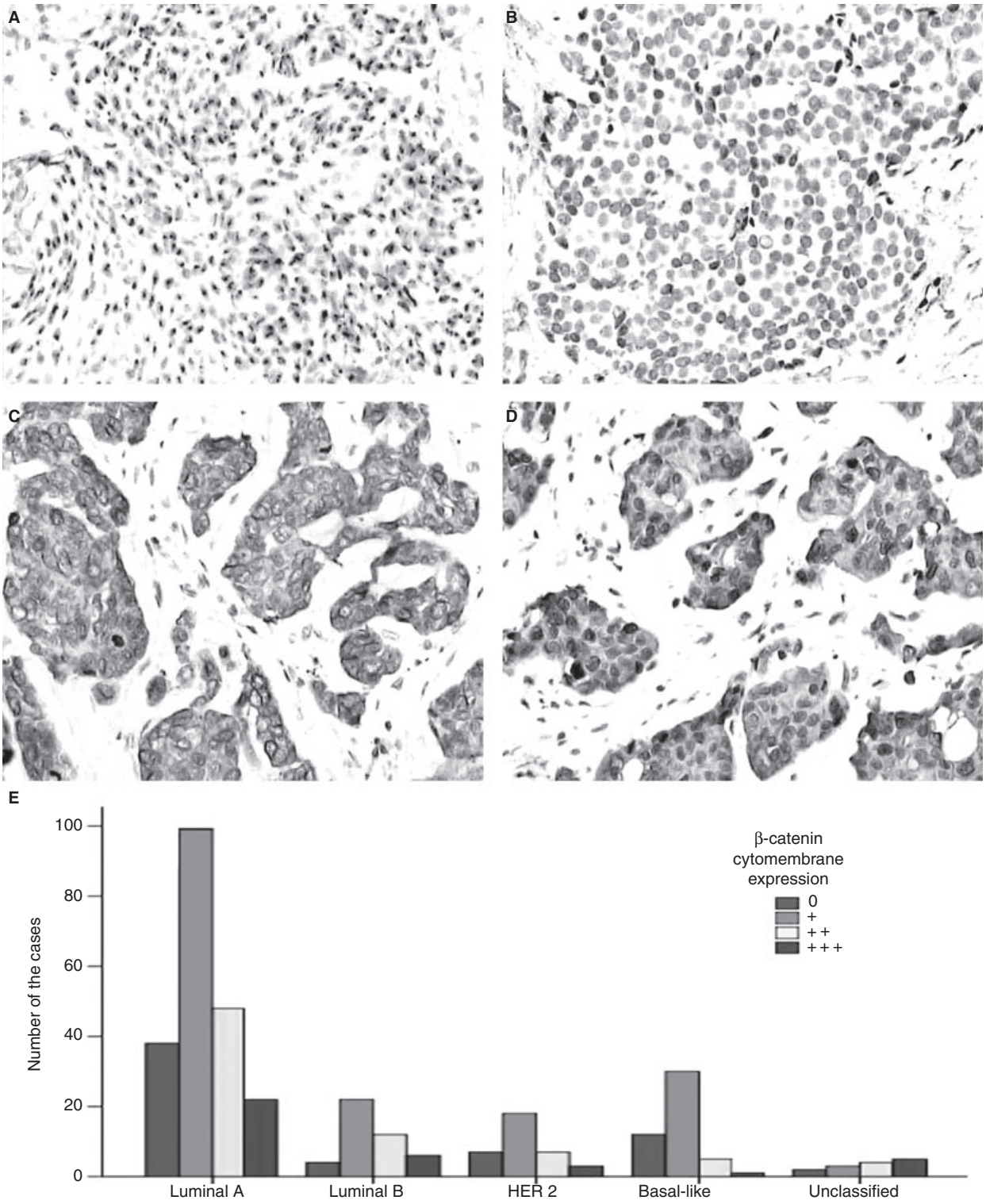

Figure 1. **A**, The case was assessed as having high-level amplification of the *HER2* gene by chromogenic *in-situ* hybridization (CISH). **B**, The case was assessed as having no amplification of the *HER2* gene by CISH. **C**, Cytokeratin (CK)5/6 was expressed in the cytomembrane and cytoplasm of tumour cells in the basal-like subtype (×200). **D**, Epidermal growth factor receptor (EGFR) was expressed in the cytoplasm of tumour cells in the basal-like subtype (×200). **E**, The cytomembrane expression of β-catenin was significantly different in the five molecular subtypes of IDC. It showed high-level expression in the luminal A subtype, but low-level expression in the basal-like and HER-2/*neu* subtypes.

manufacturer's instructions, to extract RNA. Extracted RNA (1 μg) was used for first-strand synthesis with Moloney murine leukaemia virus (Promega, Madison, WI, USA) and oligo-$(dT)_{18}$ at 75°C for 5 min, and then at 42°C for 60 min. PCR amplification was performed in a 25-μl reaction with Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 2 μl of the first-strand cDNA synthesis mixture as template. The primers sequences for *Nek2* were as follows: FP, 5′-TTGACCGGACCAATACAACA-3′; RP, 5′-CAGGAAAACATTGGCTGGTT-3′ (224 bp). Amplified products were purified with a PCR purification kit (Qiagen, Crawley, UK). To avoid contamination with genomic DNA, PCR primers spanned at least one intron of the *Nek2* gene. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control (127 bp), and its primer sequences were As follows: FP, 5′-GGTGG TCTCCTCTGACTTCAACA-3′; and RP, 5′-GTTGCTGT AGCCAAATTCGTTGT-3′. Twenty-nine and 24 cycles of PCR amplification were used for *Nek2* and *GAPDH*, respectively.

PCR products were analysed on a 2% agarose gel and visualized after staining with ethidium bromide. Each RT-PCR assay was repeated at least once for confirmation. The intensity of a band was quantitated with image analysis software (Gel-Pro 4400 image system; Bingyang Technology Co., Ltd., Beijing, China), and the ratio of *Nek2* signal to *GAPDH* signal was calculated for each normal and tumour sample. The ratio for a tumour was then divided by the ratio for matched normal tissue to indicate the relative level of expression in a tumour, and increased expression was defined when such a value was higher than 2.

STATISTICAL ANALYSIS

SPSS 15.0 (Chicago, IL, USA) was used for statistical analysis. The Mann–Whitney *U*-test, Kruskal–Wallis test and chi-square test were performed for group comparisons, and correlations between two variables were evaluated with Spearman's rank correlation test. *P*-values <0.05 were considered to be statistically significant in all of the statistical analyses.

Results

PROTEIN EXPRESSION OF NEK2

As compared with normal tissue (Figure 2A), expression of Nek2 was increased in pure DCIS (Figure 2B), concomitant DCIS (Figure 2C), and IDC (Figure 2D,E) ($P < 0.01$; Table 2). There were correlations between Nek2 cytoplasmic expression and histological grade ($P = 0.000$; Table 3) and tumour size ($P = 0.001$; Table 3), as well as Ki67 ($P = 0.000$; Table 4), in IDC. However, differences in cytoplasmic reactivity in the different age, TNM stage, lymph node metastasis, ER, PR, HER2, molecular subtype, vascular endothelial growth factor and p53 groups were not observed ($P > 0.05$; data not shown). Nek2 nuclear expression was 62/348 (17.82%) in IDC, 28/137 (20.44%) in concomitant DCIS, and 12/60 (20.00%) in pure DCIS. The expression of Nek2 showed no correlation with clinicopathological parameters in concomitant DCIS and pure DCIS ($P > 0.05$; data not shown).

MRNA EXPRESSION OF *NEK2*

Semiquantitative RT-PCR was performed to evaluate the mRNA expression of *Nek2* in 50 pairs of breast cancer specimens and matched normal breast tissues (Figure 2F). The mRNA level of *Nek2* in cancer specimens was elevated in 29/50 (58%) samples, whereas expression of *Nek2* was absent in 9/29 (31.03%) normal breast tissues from the matched cases. Representative RT-PCR results for six pairs of breast cancer samples are shown in Figure 2F. The relative ratio of *Nek2* signal was higher than 2 in 29 pairs, 1–2 in 15 pairs, and lower than 1 in six pairs. Statistical analysis showed that, as compared with matched normal tissue, *Nek2* mRNA expression was significantly increased in IDC ($\chi^2 = 49.000$, $P = 0.000$; data not shown).

THE ASSOCIATION OF NEK2, β-CATENIN, AND KI67

Nek2 and β-catenin cytoplasmic expression were both positively correlated with Ki67 ($P = 0.000$; Table 4). Nek2 cytoplasmic expression was associated with β-catenin cytoplasmic expression in pure DCIS ($r = 0.292$, $P = 0.001$; data not shown) and concomitant DCIS ($r = 0.330$, $P = 0.000$, data not shown), and a similar relationship could be seen in IDC ($r = 0.358$, $P = 0.000$; Table 5).

EXPRESSION OF β-CATENIN

As compared with normal tissue (Figure 3A), β-catenin cytoplasmic expression was increased in pure DCIS (Figure 3B), concomitant DCIS (Figure 3C), and IDC (Figure 3D,E), but β-catenin cytomembrane reactivity was decreased ($P = 0.015$; Table 2). β-Catenin membrane expression was correlated remarkably with grade ($\chi^2 = 84.451$, $P = 0.000$; Table 6), TNM stage ($\chi^2 = 18.487$, $P = 0.000$; Table 6), node status ($Z = -2.714$, $P = 0.007$, Table 6), ER ($Z = -4.792$,

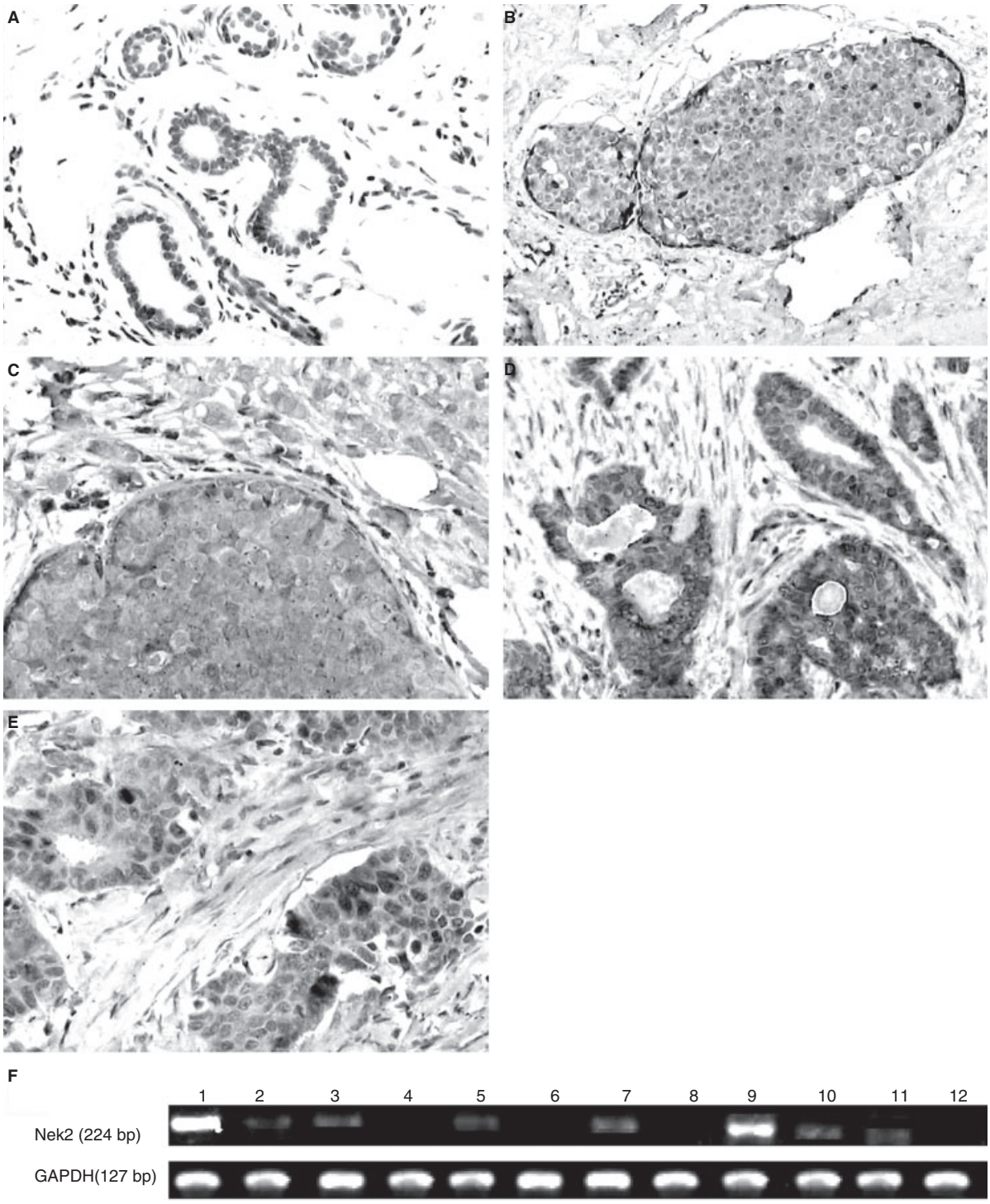

Figure 2. Immunohistochemical localization of NIMA-related kinase 2 (Nek2) in normal tissues and tumour tissues. Nek2 was expressed in the cytoplasm and/or nucleus of tumour cells. **A**, Nek2 expression in normal tissue (×200). **B**, Nek2 expression in pure ductal carcinoma *in situ* (DCIS) (×200). **C**, Nek2 expression in concomitant DCIS (×200). **D**, Nek2 reactivity in low-grade invasive ductal carcinoma (IDC) (×200). **E**, Nek2 expression in high-grade IDC (×200). As compared with normal tissue (**A**), expression of Nek2 was increased in IDC (**D**,**E**), concomitant DCIS (**C**), and pure DCIS (**B**), and there were differences between different grades of IDC (**D**,**E**). **F**, Increased expression of Nek2 (224 bp) in primary breast tumours. Representative results of reverse transcriptase polymerase chain reaction analysis of Nek2 expression in primary tumours. Lanes 1, 3, 5, 7, 9 and 11: acancer samples. Lanes 2, 4, 6, 8, 10 and 12: matched adjacent normal breast tissues. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (127 bp) served as an internal control.

Table 2. Positive expression of NIMA-related kinase 2 (Nek2) and β-catenin in invasive ductal carcinoma (IDC), concomitant ductal carcinoma *in situ* (DCIS), pure DCIS and normal tissue

	Normal		Pure DCIS		Concomitant DCIS		IDC			
	–	Positive (+,++, +++)	–	Positive (+,++, +++)	–	Positive (+,++, +++)	–	Positive (+,++, +++)	χ^2	*P*
Nek2, cytoplasm, *n* (%)	131 (95.62)	6 (4.38)	19 (31.66)	41 (68.33)	40 (29.20)	97 (70.80)	39 (28.47)	98 (71.52)	170.650	0.000
Nek2, nucleus, *n* (%)	135 (98.57)	2 (1.46)	48 (80.00)	12 (20.00)	109 (79.56)	28 (20.44)	116 (84.67)	21 (15.33)	25.909	0.000
β-Catenin, cytomembrane, *n* (%)	2 (1.46)	135 (98.54)	5 (8.33)	55 (91.67)	13 (9.49)	124 (90.51)	15 (10.95)	122 (89.05)	10.458	0.015
β-Catenin, cytoplasm, *n* (%)	117 (85.40)	20 (14.59)	3 (5.00)	57 (95.00)	8 (5.84)	129 (94.16)	1 (0.72)	136 (99.27)	327.218	0.000

Table 3. Comparison of NIMA-related kinase 2 (Nek2) cytoplasmic immunoreactivity, histological grade and tumour size in invasive ductal carcinoma

	Nek2					
	–	+	++	+++	χ^2	*P*-value
Histological grade						
I	23	27	13	7	17.833	0.000
II	45	77	35	9		
III	14	45	39	14		
Tumour size (mm)						
≤20	29	35	10	5	14.336	0.001
20–50	53	97	69	23		
>50	0	17	8	2		

P-values were calculated with the Kruskal–Wallis test.

Table 4. The association of NIMA-related kinase 2 (Nek2) cytoplasmic and β-catenin cytoplasmic expression with Ki67 in invasive ductal carcinoma

	Ki67							
	–	+	++	+++	χ^2	*P**	*r*	*P***
Nek2								
–	4	30	40	8	30.375	0.000	0.284	0.000
+	6	17	123	3				
++	2	13	55	17				
+++	0	4	13	13				
β-Catenin								
–	0	6	9	0	42.178	0.000	0.239	0.000
+	12	12	58	2				
++	0	32	126	19				
+++	0	14	38	20				

**P*-values were calculated with the Kruskal–Wallis test; ***P*-values were calculated with Spearman's rank correlation test.

$P = 0.000$; Table 6) and molecular subtype ($\chi^2 = 16.169$, $P = 0.003$; Figure 1E; Table 1) in IDC. The β-catenin cytoplasmic reactivity was correlated with TNM stage ($\chi^2 = 16.895$, $P = 0.000$; data not shown) and Ki67 in IDC ($\chi^2 = 42.178$, $P = 0.000$; Table 4). There was no correlation with clinicopathological parameters in pure DCIS and concomitant DCIS ($P > 0.05$; data not shown). Furthermore, β-catenin

Table 5. The correlation of NIMA-related kinase 2 (Nek2) cytoplasmic and β-catenin cytoplasmic expression in invasive ductal carcinoma

	Nek2					
	−	+	++	+++	*r*	*P*
β-Catenin						
−	9	6	0	0	0.358	0.000
+	23	47	12	2		
++	42	80	43	12		
+++	8	16	32	16		

P-values were calculated with Spearman's rank correlation test.

cytomembrane expression was significantly different between IDC and concomitant DCIS ($Z = -5.150$, $P = 0.000$; data not shown).

Discussion

It is known that most tumour cells in human malignancies exhibit centrosome abnormalities. Importantly, centrosome aberrations[5] are correlated with or even precede the generation of aneuploidy and the acquisition of a chromosome instability phenotype in breast tumours. The deregulation of centrosome function may be a major contributory factor to cancer progression. Nek2 and β-catenin are the important centrosome regulatory factors. They were thought to be important in finding molecular mechanisms for tumorigenesis, and they may represent new targets for therapeutic intervention.

Nek2 is a serine/threonine protein kinase that is located at the centrosome and is implicated in mitotic regulation. Overexpression of Nek2[13] induces premature centrosome separation and nuclear defects, which are indicative of mitotic errors. Nek2[14,15] is involved in cell division and mitotic regulation by centrosome splitting. Hayward *et al.*[16] found that Nek2 protein expression was elevated 2–5-fold in cell lines derived from a range of human tumours, including those of the breast, ovary, and prostate. In another study, Hayward and Fry[17] examined 20 high-grade IDCs by immunohistochemistry, and observed a remarkable degree of Nek2 up-regulation in 16 samples as compared with normal tissue. However, the tumours examined were all high-grade, which means that the expression of Nek2 in different grades of IDC has not been reported. Our study showed that Nek2 expression in DCIS and IDC was higher than that in normal tissue; furthermore, Nek2 cytoplasmic expression was correlated significantly with histological grade and tumour size. Nek2 mRNA is elevated markedly in breast carcinoma. The up-regulation in concomitant DCIS indicates that alteration of Nek2 protein levels may occur in breast tumours before invasion and metastasis. As is well known, high grade and large tumour size are poor prognostic indicators. Therefore, this finding implies that the more the up-regulation of Nek2 expression, the poorer the prognosis may be. A possible mechanism[18] for this is that Nek2 regulates centrosome cohesion and separation through phosphorylation of structural components of the centrosome, and aberrant Nek2 can lead to aneuploid defects of cancer cells by regulating centrosome separation. Liu *et al.*[19] believe that elevation of Nek2 expression may contribute to chromosome instability and promote aneuploidy by disrupting the control of the mitotic checkpoint.

Our study showed Nek2 in carcinoma cells were cytoplasm and/or nuclear expression in IDC and DCIS. From this, we inferred that Nek2 has both nuclear and cytoplasmic functions. This finding bears some similarities to the results of Hayward *et al.*[16] The mechanism for this may be related to the fact that Nek2 has three major splice variants[1] (Nek2A, Nek2B, and Nek2C), and they exhibit a remarkable difference in distribution. Because the existence of a functional bipartite nuclear localization sequence (NLS) that spans the splice site leading to Nek2C has a strong NLS. Nek2A has a weak NLS, and Nek2B has no NLS. Nek2C is mainly located in the nucleus, Nek2B is mainly located in the cytoplasm, and Nek2A is evenly distributed between the nucleus and the cytoplasm.

Importantly, a correlation was found between Nek2 and Ki67 (a marker of cell proliferation). Therefore, we infer that cells showing an inappropriately high level of expression of Nek2 are proliferating. Some data[20] have shown that Nek2 small interfering RNA (siRNA) has an inhibitory effect on cellular proliferation in breast carcinoma. Nek2 siRNA suppressed anchorage-independent growth, suggesting that breast carcinoma cells have a specific dependence on Nek2 for their tumorigenic growth; this was described by Weinstein[21] as 'oncogene addiction'. This provided a strong rationale for further investigation of Nek2 as a new breast carcinoma therapy target. It may become a new antitumour drug target[20,22] in the future.

β-Catenin is a biologically important molecule, playing critical roles[23] in cell–cell adhesion with E-cadherin as a complex at the plasma membrane. Research[2] has shown that β-catenin is located and functions at the centrosome, which represents a

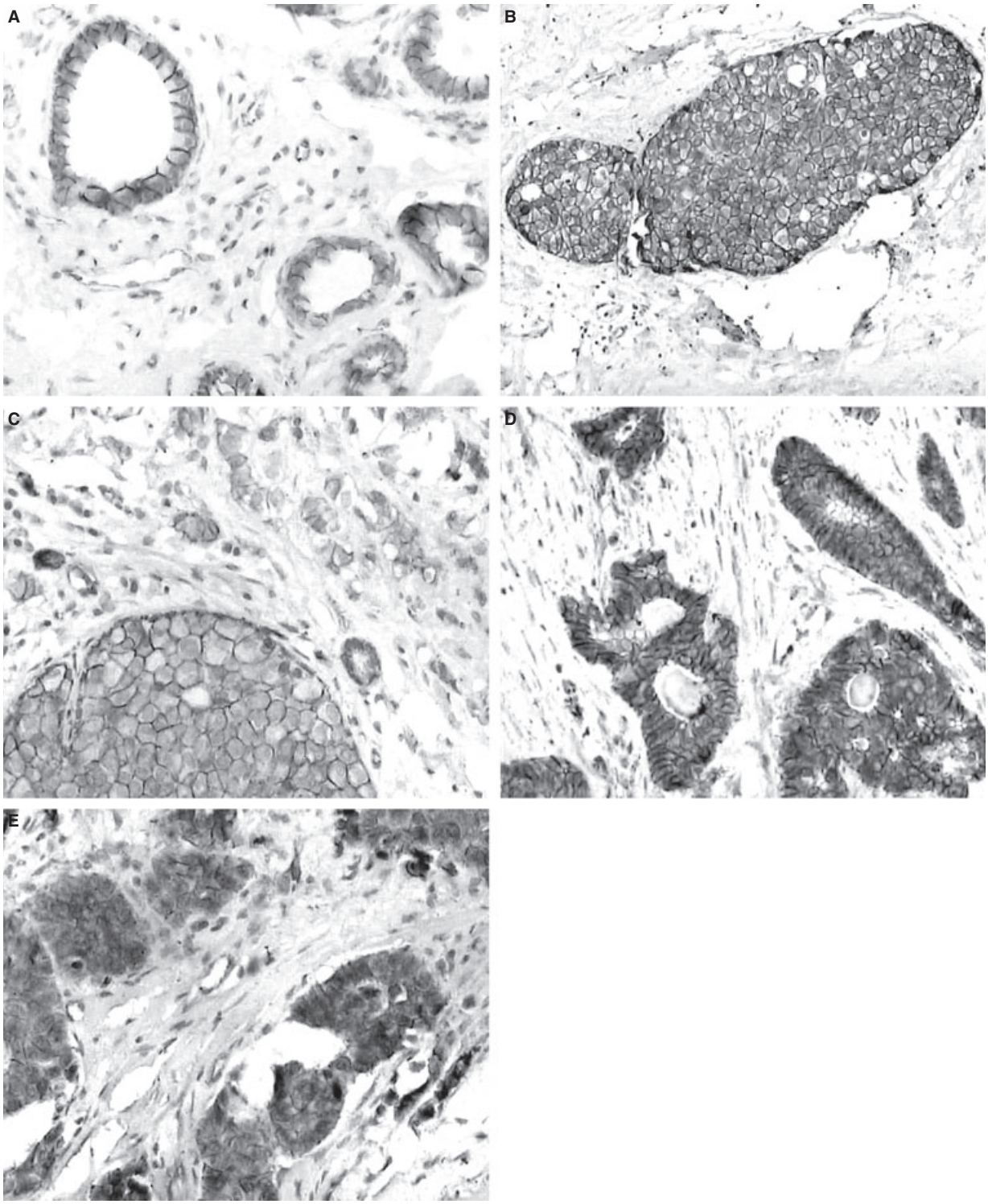

Figure 3. Immunohistochemical localization of β-catenin in normal and tumour tissues. β-Catenin was expressed in the cytomembrane and cytoplasm of tumour cells, whereas it was mainly expressed in the cytomembrane of normal cells. **A**, β-Catenin expression in normal tissue (×200). **B**, β-Catenin expression in pure ductal carcinoma *in situ* (DCIS) (×200). **C**, β-Catenin expression in concomitant DCIS (×200). **D**, β-Catenin reactivity in low-grade invasive ductal carcinoma (IDC) (×200). **E**, β-Catenin expression in high-grade IDC (×200). As compared with normal tissue (**A**), β-catenin cytoplasmic expression was increased in pure DCIS (**B**), concomitant DCIS (**C**), and IDC (**D**,**E**), whereas β-catenin cytomembrane reactivity was decreased.

Table 6. Comparison of β-catenin cytomembrane immunoreactivity and some clinicopathological characteristics in invasive ductal carcinoma

	β-Catenin					
	−	+	++	+++	χ^2/Z	*P*
Histological grade						
I	0	20	23	27	84.451	0.000*
II	34	79	43	10		
III	29	73	10	0		
TNM stage						
I	2	19	20	9	18.487	0.000*
II	39	77	35	18		
III	22	76	21	10		
Lymph node metastasis						
−	21	70	37	22	−2.714	0.007**
+	42	102	39	15		
ER						
−	36	71	24	3	−4.792	0.000**
+	27	101	52	34		

ER, oestrogen receptor.
**P*-values were calculated with the Kruskal–Wallis test; ***P*-values were calculated with the Mann–Whitney *U*-test.

new subcellular location. In our study, β-catenin cytoplasmic expression in DCIS and IDC was higher than that in normal tissue, and β-catenin cytomembrane reactivity was lower. The change in concomitant DCIS indicates that alteration of β-catenin levels may occur in breast tumours before invasion and metastasis. Furthermore, β-catenin cytomembrane expression was correlated significantly with histological grades, TNM stage, node metastasis and ER expression. β-Catenin cytoplasmic expression is related to TNM stage and Ki67. β-Catenin appears to be located mainly in the membrane in normal cells, which is consistent with the results of Sadot *et al.*[24] These results hint that β-catenin cytomembrane expression is a good prognostic sign, but cytoplasmic expression is a poor prognostic sign. One of the possible explanations for this is that cytoplasmic overexpression of β-catenin disrupts both the organization of MTs at the centrosome and the capture and tethering of MTs at the adherens junction.[25] Another explanation[3] is that phosphor-β-catenin accumulates in the centrosome instead of being degraded immediately after its phosphorylation, and transcriptional up-regulation of cell proliferation genes such as those encoding cyclin D1 and c-Myc contributes to the oncogenic function of β-catenin. This is consistent with our results showing β-catenin cytoplasmic expression to be related to the proliferation-associated nuclear antigen Ki67.

In addition to transcriptional control, β-catenin may also contribute to tumorigenesis[3] by causing centrosomal abnormalities and genetic instability. Some results[2] have identified β-catenin as a component of the linker between centrosomes, and have defined a new function for β-catenin as a key regulator of centrosome separation in mitosis. β-Catenin is a negative regulator[26] of centrosome cohesion, and it is also essential for the establishment of a bipolar mitotic spindle. A recent study[27] found that, in cooperation with other factors, β-catenin can also lead to unequal cell division by regulating the formation of an MT bundle that shortens and pulls the centrosome towards a subcellular cortical structure known as the centrosome-attracting body. Furthermore, overexpression of β-catenin may increase[25] the numbers of γ-tubulin puncta in the centrosome region, and lead to impaired[3] MT anchoring at centrosomes. Some data[28] have indicated that it could be a new antitumour drug target.

Breast carcinoma is now regarded as a heterogeneous disease, classified into five molecular subtypes (luminal A, luminal B, basal cell-like, normal-like, and HER-2/*neu*). These five subtypes of breast tumour are characterized by distinct clinical and pathological features, and respond in accordingly distinct manners to chemotherapy. On comparison of the prognosis of tumours within each of the different subtypes, it was shown[29] that basal cell-like or HER-2/*neu* tumours showed more aggressive clinical behaviour, whereas luminal A tumours were associated with a better prognosis. Our study showed that the cytomembrane expression of β-catenin was significantly different in the five molecular subtypes. It showed high-level expressed in the luminal A subtype, but low-level expression in the basal cell-like and HER-2/*neu* subtypes. This result indicated that the expression of β-catenin could be an effective prognostic factor.

In our study, we found that Nek2 cytoplasmic expression was correlated with β-catenin cytoplasmic expression. The following assumptions may explain our result. Hayward and Fry[17] think that Nek2 may trigger centrosome separation at the onset of mitosis, by phosphorrylation of multiple linker components, and Bahmanyar[2] proposes that β-catenin is a Nek2 substrate involved in centrosome separation. In mitosis, when Nek2 activity increases, β-catenin is located at centrosomes at spindle poles, and is phosphorylated by

Nek2 in a complex that holds the centrosomes together. When Nek2 activity is abnormally increased, it can disrupt correct centrosome separation. Therefore, Nek2 and β-catenin are the linker components of centrosomes, and they are the key regulators in mitotic centrosome separation.

In conclusion, our results indicate that the abnormal expression of Nek2 and β-catenin in concomitant DCIS and IDC might play an important role in tumour progression directly or indirectly, and might be one of the molecular mechanisms of abnormal tumour proliferation. This may provide a new approach to explore the mechanisms of breast tumour genesis. The findings also reveal the potential of Nek2 and β-catenin as new targets for therapeutic intervention.

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