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Expression of cell-cycle regulatory proteins BUBR1, MAD2, Aurora A, cyclin A and cyclin E in invasive ductal breast carcinomas

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Summary. Cyclin A, cyclin E, BUBR1, MAD2 and Aurora A are all cell-cycle regulatory proteins and have been proven to play crucial roles in carcinogenesis. However, their expression patterns in invasive ductal breast carcinoma (IDBC) are controversial and unclear. In this study, we examined the expression status of these candidate proteins in a set of 117 invasive ductal carcinomas, and evaluated their associations with known clinicopathological parameters and the expressions of estrogen receptor, progesterone receptor, Ki-67 and Her-2. Univariate and multivariate data analyses both displayed that positive BUBR1 expression was associated with a high Ki-67 labeling index, and negative MAD2 expression was associated with Her-2 overexpression. Positive BUBR1 expression was also associated with a high histological tumor grade in univariate analysis, but not in multivariate analysis. In addition, high Aurora A expression was weakly associated with lymph node metastasis, and cyclin A was strongly associated with the expression of cyclin E in both univariate and multivariate models. In conclusion, this study suggests that evaluation of BUBR1, MAD2 and Aurora A expression levels is likely to improve accuracy of prognostic predictions in IDBC.

Key words: Ductal breast carcinoma, BUBR1, MAD2, Aurora A

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Introduction

Breast cancer is the most frequent malignancy in women worldwide, and its incidence has increased rapidly in China and other Asian countries over the last two decades. A unifying feature of cancer is uncontrolled cell growth. The cell cycle in eukaryotes consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis), which is a series of coordinated events. Defects in this process, such as misregulation of DNA amplification and centrosome duplication, as well as mitotic errors, may result in unscheduled proliferation, genomic and chromosomal instability, and contribute to aneuploidy and carcinogenesis (Malumbres and Barbacid, 2007; Schmit and Ahmad, 2007).

A variety of cell-cycle regulatory proteins, such as cyclin A, cyclin E, Aurora A, MAD2 (mitosis arrest-deficient 2) and BUBR1 (budding uninhibited by benzimidazoles 1, also known as BUB1B), are involved in DNA and/or centrosome duplication or mitosis. Their aberrant expressions or gene mutations may play pivotal roles in tumor development and progression (Schmit and Ahmad, 2007; Malumbres and Barbacid, 2007). Cyclin A and cyclin E are cell cycle regulators, having important functions in both the process of DNA synthesis and centrosome duplication (Malumbres and Barbacid, 2007). There have been reports about overexpressions of the two proteins in breast cancer (Bostrom et al., 2009), while data on their associations with clinicopathological parameters are conflicting (Keyomarsi et al., 2002; Kuhling et al., 2003; Berglund and Langberg, 2006; Ahlin et al., 2009). BUBR1 and MAD2 are components of the spindle-assembly checkpoint (SAC), which plays a crucial role in monitoring the process of cell mitosis and is essential to

maintain genomic stability during cell division (Musacchio and Salmon, 2007). Although gene mutations and reduced expression in BUBR1 or MAD2 have been reported to cause aneuploidy in cancer cells by compromising the mitotic checkpoint, resulting in chromosome mis-segregation (Li and Benezra, 1996; Percy et al., 2000; Michel et al., 2001; Hanks et al., 2004), overexpressions of BUBR1 and MAD2 have also been implicated in tumorigenesis and progression (Li and Zhang, 2004; Yuan et al., 2006). Aurora A, a mitotic kinase, is mainly involved in centrosome duplication, mitotic entry and spindle assembly (Vader and Lens, 2008). Overexpression of Aurora A may lead to centrosome amplification, chromosomal instability and transformation in mammalian cells (Zhou et al., 1998). Increased expression of Aurora A was observed in several types of carcinomas, including breast cancer (Vader and Lens, 2008). Nadler Y found significant correlations of Aurora A overexpression with high nuclear grade, high Her2/neu and shortened overall survival (2008), while Royce et al. (2004) demonstrated no association between Aurora A expression and survival. In contrast, Hoque et al. (2003) even revealed a decreased expression of Aurora A in invasive breast cancer when compared with adjacent carcinoma in situ.

Such controversial results in the literature demand further studies on the associations of these cell-cycle related proteins with clinicopathological parameters. In this retrospective study, we investigated the expressions of cyclin A, cyclin E, BUBR1, MAD2 and Aurora A in a series of 117 invasive ductal breast carcinomas (IDBC). Furthermore, we evaluated potential associations of these protein expressions with known clinico-pathological parameters and the expressions of estrogen receptor (ER), progesterone receptor (PR), Ki-67 and Her-2. In addition, we studied the associations between the expressions of these proteins.

Materials and methods

Patients

Tumor samples were obtained from 117 patients who underwent surgery for treatment of invasive ductal carcinoma at the Third Hospital of Peking University, Beijing, China, in a period between the years 2007 and 2008. These patients underwent modified radical mastectomy with axillary lymph node dissection. No detectable distant metastasis was found at the time of surgery. The diagnosis of the primary pathology was confirmed in H&E staining. Tumor grades were assessed according to the Nottingham modification of the Bloom-Richardson histologic grading scheme. Tumor size and axillary lymph nodes status were obtained from the pathology reports. Clinical information was derived from medical records. The immunostainings of molecular markers ER, PR, Her-2 and Ki-67 were performed in routine tests and were re-assessed by us for clarity and validation.

Immunohistochemical staining

For the detection of cyclin A, cyclin E, BUBR1, MAD2 and Aurora A, formalin-fixed and paraffin-embedded tumor tissues were sectioned and immunostained according to the protocol described below. After deparaffinization and rehydration, slices were pretreated with citrate buffer (pH 6.0) in a pressure cooker for 2 minutes for antigen retrieval. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 15 minutes. Following a blocking step for non-specific staining, the sections were incubated with primary antibodies at room temperature for an hour. After that, the EnVision™ /HRP (Dako) was added to the slices and then peroxidase reactivity was visualized using DAB substrate Kit (Dako). Finally the sections were counterstained with hematoxylin and mounted. Positive control sections were included in each staining batch. Negative control sections were incubated with normal mouse or rabbit serum instead of the primary antibody. Table 1 gives information about the used antibodies.

Evaluation of immunohistochemistry

Staining results for each antibody were interpreted by two of the authors independently. Discordant cases were reviewed and agreed upon before data were statistically analyzed. For each sample, at least five fields (x 400) and more than 500 cells were analyzed.

For the immunostaining of cyclin A, only tumor cells with nuclear staining were scored as positive. As normal breast epithelium usually shows less than 2% positivity (Elayat et al., 2009), only the lesions showing

Table 1. Antibodies, their manufacturers, clonality and working conditions.

Antibody	Manufacturer	Clonality	Dilution
Cyclin A	Santa Cruz Biotechnology (H-432), USA	Rabbit polyclonal	1:1000
Cyclin E	Santa Cruz Biotechnology (M-20), USA	Rabbit polyclonal	1:1000
BUBR1	BD transduction (612503), USA	Mouse monoclonal	1:300
Aurora A	Abcam (ab12875) Cambridge, UK	Rabbit polyclonal	1:60
MAD2	BD transduction (610679), USA	Mouse monoclonal	1:200

more than 2% nuclear staining were considered to be positive. The expression of cyclin E was evaluated similarly to cyclin A, but only cases with more than 5% nuclear staining were considered positive (Scott and Walker, 1997). For BUBR1 and MAD2, we used 10% as the cut-off point (Hisaoka et al., 2008). BUBR1 expression was evaluated as positive if more than 10% tumor cells showed nuclei or cytoplasm staining. MAD2 expression was defined as positive if more than 10% tumor cells showed nuclear staining (Hisaoka et al., 2008). As Aurora A was stained in nearly all of the breast cancer cells as well as in normal breast epithelial cells (weak staining), Aurora A expression on tumor cells was evaluated according to the immunostaining intensity. The staining intensity was rated as follows: score 0: no staining; score 1: weak intensity (equivalent to normal control epithelium); score 2: moderate intensity; score 3: strong intensity (Lee et al., 2009). Score 0-1 and score 2-3 (staining signal stronger than that in normal control epithelium) were defined as low expression and high expression, respectively.

ER and PR were evaluated as positive if more than 5% of the cells showed nuclear staining. Her-2 immunostaining on tumor cells was evaluated as suggested by ASCO/CAP Guideline (Wolff et al., 2007). Her-2 staining of 3+ (uniform, intense membrane staining of >30% of invasive tumor cells) was considered as positive or high expression. Ki-67 labeling index was defined as the percentage of tumor cells displaying nuclear immunoreactivity.

Statistics

The statistical analyses were carried out using the SAS system for windows V8 program. The associations between expression of biomarkers (cyclin A, cyclin E, BUBR1, Aurora A and MAD2) and clinicopathological parameters were tested in univariate models with one way ANOVA and in multivariate models with linear regression. A p-value <0.05 was considered statistically significant.

Results

Clinicopathological characteristics of the patients and tumor samples

The age of the patients at diagnosis ranged from 33 to 83 years. The median age was 54 years. No patient had detectable distant metastases at the time of surgery. The majority of cases (43%) had grade II disease, and the tumor diameter was larger than 2cm in 62% of the patients. ER and PR were positively stained in 64% and 76% of the cases, respectively. Her-2 was overexpressed in 34% of the cases. The clinicopathological characteristics of the patients and staining status of the

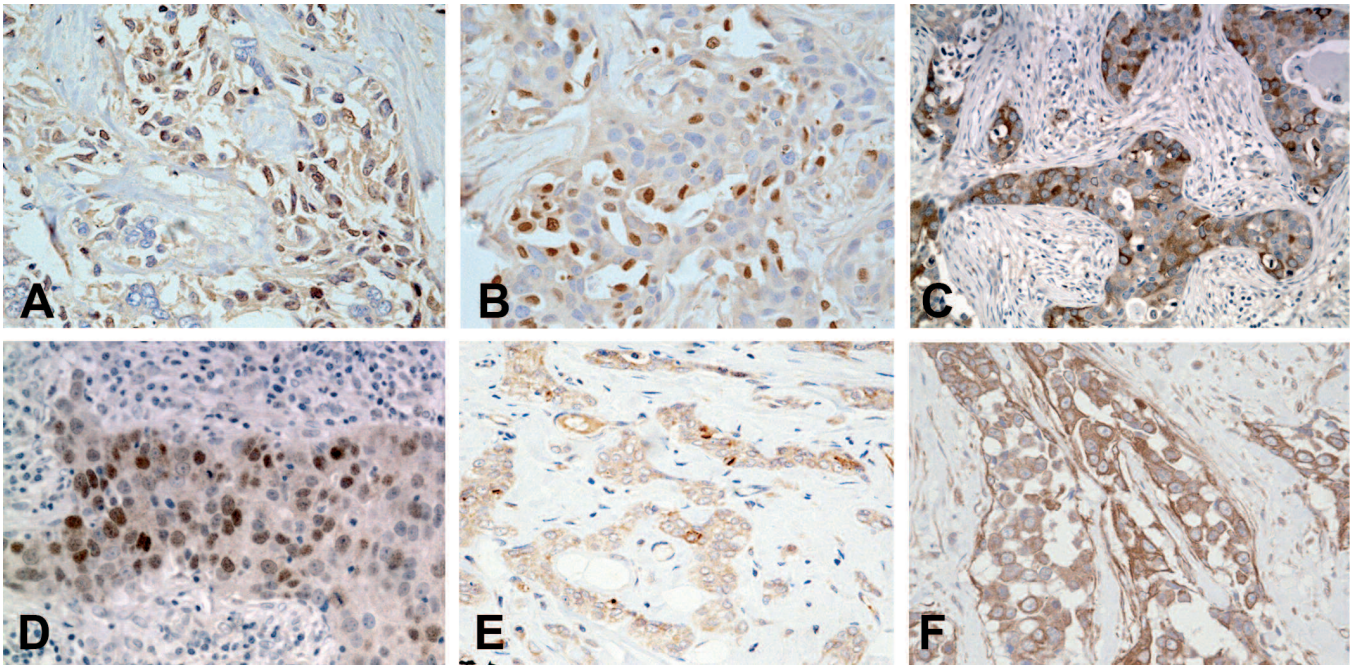

Fig. 1. Expressions of various molecular markers in breast cancer. Positive expressions of cyclin A **(A)** and cyclin E **(B)** in cancer cell nuclei. Positive expression of BUBR1 in cancer cell cytoplasm **(C)**. Positive expression of MAD2 in cancer cell nuclei **(D)**. Low and high expressions of Aurora A in cancer cell cytoplasm (**E and F**, respectively). x 40

molecular markers are summarized in table 2.

Immunohistochemical staining

Cyclin A and cyclin E are members of a cell cycle protein family and only nuclear staining for these two proteins were considered positive (Fig. 1A,B). Normal breast epithelium cells were negative for cyclin A and cyclin E staining. A faint background reaction in the connective tissue and in the cytoplasm of cancer cells was seen in some cases, but it never posed problems in the evaluation of true immunoreactivity. In this study, expressions of cyclin A and cyclin E was positive in 26% (30/117) and 31% (36/117) of the cases, respectively (table 2). The expression of cyclin A and cyclin E was neither associated with known clinicopathological parameters, nor with the expression status of ER, PR, Her-2 and Ki-67 ($p>0.05$) (Table 2).

BUBR1 showed no staining in normal breast epithelium under the conditions used in this study, whereas it demonstrated a heterogenous staining pattern in breast carcinoma cells. Staining was not only confined to the nucleus, but also presented diffusely in the cytoplasm (Fig 1C). Interestingly, we found that all of the tumor cells during mitosis were positive for BUBR1 expression. BUBR1 staining occurred more often in the younger age group (≤50 years) than in the older age group (>50 years). Staining for BUBR1 is more prevalent in grade 2 (44%) and grade 3 (46%) cases than in grade 1 cases (16%). In addition, BUBR1 expression appeared more often in high Ki-67 index group than in low Ki-67 index group (69% vs 27%). In univariate analysis with one way ANOVA, BUBR1 expression was inversely associated with age ($p=0.0056$), and positively associated with histological grade ($p=0.022$) and Ki-67 ($p<0.0001$). In multivariate analysis with linear regression models, including all the parameters listed in table 2, BUBR1 expression was only associated with age and Ki-67 ($p=0.0019$ and $p<0.0001$, respectively), indicating that these associations may be independent of the other parameters included in the analyses. However, multivariate analysis did not show any correlation

Table 2. Associations of Cyclin A, Cyclin E, BUBR1, MAD2 and Aurora A with clinicopathological parameters and expressions of ER, PR, Ki-67 and Her-2.

	Total number	cyclin A			cyclin E			BUBR1			MAD2			Aurora A		
		-	+	*P** value	-	+	*p* value	-	+	*p* value	-	+	*p* value	Low	High	*p* value
Age (yrs)																
≤50	49	36	13		35	14		23	26		24	25		15	34	
>50	68	51	17	NS	46	22	NS	49	19	0.0056	36	32	NS	13	55	NS
Histological grade																
Grade 1	26	22	4		21	5		22	4		16	10		7	19	
Grade 2	50	35	15		32	18		28	22		25	25		8	42	
Grade 3	44	30	11	NS	28	13	NS	22	19	0.022	19	22	NS	13	28	NS
Tumor size																
≤2cm	43	31	12		29	14		26	17		18	25		7	36	
2-5cm	62	47	15		42	20		41	21		34	28		20	42	
≥5cm	10	7	3	NS	8	2	NS	5	5	NS	8	2	NS	1	9	NS
Lymph nodes																
negative	57	43	14		40	17		37	20		25	32		18	39	
positive	56	41	15	NS	39	17	NS	33	23	NS	34	22	NS	9	47	0.0539
Clinical Stage																
Stage I	27	19	8		17	10		16	11		9	18		5	22	
Stage II	70	53	17		49	21		47	23		39	31		21	49	
Stage III	18	13	5	NS	14	4	NS	8	10	NS	12	6	NS	2	16	NS
Estrogen receptor																
-	42	33	9		30	12		23	19		21	21		13	29	
+	75	54	21	NS	51	24	NS	49	26	NS	39	36	NS	15	60	NS
Progesterone receptor																
-	28	20	8		17	11		13	15		15	13		8	20	
+	88	66	22	NS	63	25	NS	58	30	NS	44	44	NS	20	68	NS
Ki-67																
<40%	85	68	17		59	26		61	24		48	37		20	65	
≥40%	28	18	10	NS	20	8		8	20	<0.0001	10	18		7	21	
Her-2																
0-++	77	58	19		53	24		51	26		46	31		19	58	
+++	40	29	11	NS	28	12	NS	21	19	NS	14	26	0.0108	9	31	NS

P* values were calculated by univariate models with one way ANOVA. NS, not significant.

between BUBR1 and histological grade, indicating that this association may be affected by other parameters.

MAD2 was mainly stained in nuclei, and sometimes could be seen in both nuclei and cytoplasm (Fig 1D). MAD2 was negative in normal breast epithelium, and positive in 49% of ductal carcinoma cases. MAD2 expression was inversely associated with Her-2 expression in both univariate (p=0.0108) and multivariate analyses (p=0.0019), indicating that the association between MAD2 and Her-2 expression may be independent of the other parameters included in the analyses. MAD2 expression was not associated with other parameters (p>0.05) as shown in table 2.

In this study, the subcellular localization of Aurora A was cytoplasmic with sparse nuclear staining. Aurora A was stained in nearly all of the breast cancer cells (Fig. 1E,F), as well as in normal breast epithelial cells, which showed weak staining. High expression of Aurora A occurred more often in lymph node positive group than in lymph node negative group (84% vs 68%). There was a borderline association between Aurora A expression and lymph node metastasis in univariate analysis (p=0.0539). Aurora A expression was also weakly associated with lymph node metastasis in multivariate analysis (p=0.0295). Apart from that, Aurora A expression was neither associated with any other clinicopathological parameters, nor with the expression status of ER, PR, Ki-67 or Her-2 (p>0.05) (Table 2).

Associations between the expressions of these biomarkers

The associations between the expressions of these biomarkers were also examined in univariate models with one way ANOVA models (Table 3). Cyclin A positive staining occurred much more often in the cyclin E positive group than in the cyclin E negative group (59% vs 10%). The expression of cyclin A was strongly associated with cyclin E immunopositivity in tumor cells (p<0.0001). This association remained statistically significant in multivariate analyses (p<0.0001). There was no correlation between the expressions of other biomarkers (Table 3).

Discussion

Among these five cell-cycle related genes, BUBR1 and MAD2 showed the most striking results in this study. BUBR1 and MAD2 are key components of the mitotic spindle checkpoint, which delays mitosis when chromosomes are imperfectly aligned (Musacchio and Salmon, 2007). Based on such functions, it is logical to predict that mutations or underexpression of these mitotic spindle checkpoint genes may play a role in aneuploidy and carcinogenesis. Supporting this hypothesis, Baker et al reported that mutant mice with low levels of Bub1b (ortholog to human BUBR1) developed progressive aneuploidy, defected in meiotic chromosome segregation and infertility (Baker et al., 2004). Hanks et al identified truncating and missense mutations of BUBR1 in five families with mosaic variegated aneuploidy, including two with embryonal rhabdomyosarcoma (Hanks et al., 2004). Furthermore, Shin HJ revealed reduced expression of BUBR1 in colon cancer (Shin et al., 2003). However, up-regulation of BUBR1 has also been reported to be associated with very aggressive cancer phenotypes and poor prognosis. Yuan et al. (2006) evaluated expression of BUBR1 in a panel of 270 primary breast cancer samples represented on tissue microarrays, and found that increased expression of BUBR1 was correlated with high-grade breast cancer, which is consistent with our findings. Similar results were also reported in bladder, kidney and hepatocellular carcinomas (Yamamoto et al., 2007; Pinto et al., 2008; Liu et al., 2009). Scintu et al. (2007) demonstrated an increase of BUBR1 mRNA in the majority of the invasive ductal carcinomas tested, and

Table 3. Associations between the expression of Cyclin A, Cyclin E, BUBR1, MAD2 and Aurora A.

	Cyclin E			BUBR1			Aurora A			MAD2		
	-	+	*P** value	-	+	*p* value	Low	High	*p* value	-	+	*p* value
Cyclin A												
negative	72	15	<0.0001	57	30	NS	23	64	NS	46	41	NS
positive	9	21		15	15		5	25		14	16	
Cyclin E												
negative				50	31	NS	21	60	NS	43	38	NS
positive				22	14		7	29		17	19	
BUBR1												
Low							17	55	NS	42	30	NS
high							11	34		18	27	
Aurora A												
Low										15	13	NS
high										45	44	

P* values were calculated by univariate models with one way ANOVA. NS, not significant.

found that BUBR1 mRNA level was correlated with intrachromosomal instability. For the first time, in a cohort of 117 patients with IDBC, we illustrated that a high BUBR1 expression was positively associated with Ki-67 labelling index, which reflected the cell proliferation extent. Comparable results were shown in bladder and hepatocellular cancer (Yamamoto et al., 2007; Liu et al., 2009). As more and more studies are supplying evidence that an overexpression of BUBR1 correlates with a high histological tumor grade or poor survival, it can be speculated that BUBR1 expression is significant in carcinogenesis and tumor progression. In terms of MAD2, results from different studies were also contradictory. Aberrantly reduced expression of MAD2 protein has been correlated with a defective mitotic checkpoint in breast, NPC and ovary carcinoma cells (Li and Benezra, 1996; Wang et al., 2000, 2002). In this study, MAD2 expression was negative in 51% of IDBC cases, and negative MAD2 expression was correlated with Her-2 overexpression, which usually predicts poor prognosis in breast cancer. However, Yuan et al. examined the mRNA and protein levels of MAD2 by Real-Time PCR and Western blotting methods, and found that MAD2 expression was significantly higher in 83% (10/12) of breast cancer cell lines and 67% (6/9) of primary breast cancer tissues than in normal mammary epithelial cells or in normal breast tissues. Differences in detection methods, scoring criteria and sample size may contribute to these conflicting results. With regard to our finding that negative MAD2 expression was associated with Her-2 overexpression, further studies are necessary to ascertain this result.

Regarding the subcellular localization of Aurora A, Shen et al. (2009) and Burum-Auensen et al. (2007) found that Aurora A was expressed both in nuclei and in cytoplasm of tumor cells. In contrast, we found that Aurora A was localized mainly in cytoplasm with sparse nuclear staining, which is consistent with Mendiola et al. (2009) and Ogawa et al. (2008) findings. Although amplification of Aurora A gene as well as overexpressions of Aurora A mRNA and protein have been demonstrated in human breast cancers (Vader and Lens, 2008; Nadler et al., 2008), its value as a prognostic marker in breast cancer remains unclear. Royce et al. (2004) showed no association between Aurora A staining and lymph node status, hormone receptor status, tumor grade or prognosis. By contrast, Nadler et al. (2008) reported that high Aurora A expression was associated with a high HER-2/neu and shortened survival. The contradictions between those two studies could be explained by the fact that Nadler et al. investigated a much larger patient cohort (638 patients) with 15-year follow-up, including a larger proportion of node-negative cases, and used a newly developed method of automated quantitative analysis of tissue microarrays. Nadler et al. finding (2008) was indirectly supported by our own observation, showing that high Aurora A level was weakly associated with a positive lymph node status, which is synonymous with poor prognosis.

Cyclin E plays a critical role in G1/S transition and was reported to be a prognostic molecule in many breast cancer studies (Malumbres and Barbacid, 2007). Overexpression of cyclin E was strongly linked to an aggressive phenotype and poor prognosis in breast cancer (Kühling et al., 2003), but cyclin E overexpression was also demonstrated to decrease mobility and invasiveness of breast cancer cells (Berglund and Landberg, 2006). Our findings did not show any links between cyclin E and these clinicopathological parameters. Cyclin A increases in early S phase and decreases in mid–M phase, and has been considered as a proliferative marker (Malumbres and Barbacid, 2007). The overexpression of cyclin A was associated with high histological tumor grade, Ki-67 and worse prognosis for breast cancer patients (Baldini et al., 2006; Ahlin et al., 2007), whereas Kuhling et al. (2003) reported that cyclin A did not achieve statistical significance in predicting disease-specific and metastasis-free survival in lymph node negative breast carcinomas. Our results, however, only revealed an association between cyclin A and cyclin E, which is in agreement with the findings of Bostrom et al. (2009). Regarding the prognostic values of cyclin A and cyclin E, these contradictory results may be partly explained by the fact that tumors with high cyclin A or cyclin E expression may have a high proliferation rate, and would be more sensitive to chemotherapy targeted at cells in the S and M phases of the cell cycle (Huuhtanen et al., 1999). Other reasons for conflicting results in studies, focusing on associations between biomarkers and clinicopathological parameters, may be due to differences in immunostaining procedures, such as formalin fixation time, the antigen retrieval buffer and its pH, the used antibodies and the scoring criteria. Therefore, standardization of evaluation methods and scoring systems are required in future study.

In conclusion, this study demonstrates that positive immunostaining of BUBR1 is associated with a high Ki-67 labelling index, and negative expression of MAD2 is associated with Her-2 overexpression. Furthermore, high Aurora A expression is weakly correlated with a positive lymph node status. Taking the cumulative results into consideration, the combinations of different biomarkers and conventional clinicopathological parameters should improve prognostic abilities.

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