Original Article

Overexpression of centromere protein H is significantly associated with breast cancer progression and overall patient survival

Wen-Ting Liao[1,2,3], Yan Feng[1,2], Men-Lin Li[1,2], Guang-Lin Liu[1,2], Man-Zhi Li[1,2], Mu-Sheng Zeng[1,2] and Li-Bing Song[1,2]

Abstract

   Breast cancer is one of the leading causes of cancer death worldwide. This study aimed to analyze the expression of centromere protein H (CENP-H) in breast cancer and to correlate it with clinicopathologic data, including patient survival. Using reverse transcription-polymerase chain reaction and Western blotting to detect the expression of CENP-H in normal mammary epithelial cells, immortalized mammary epithelial cell lines, and breast cancer cell lines, we observed that the mRNA and protein levels of CENP-H were higher in breast cancer cell lines and in immortalized mammary epithelial cells than in normal mammary epithelial cells. We next examined CENP-H expression in 307 paraffin-embedded archived samples of clinicopathologically characterized breast cancer using immunohistochemistry, and detected high CENP-H expression in 134 (43.6%) samples. Statistical analysis showed that CENP-H expression was related with clinical stage (P = 0.001), T classification (P = 0.032), N classification (P = 0.018), and Ki-67 (P < 0.001). Patients with high CENP-H expression had short overall survival. Multivariate analysis showed that CENP-H expression was an independent prognostic indicator for patient survival. Our results suggest that CENP-H protein is a valuable marker of breast cancer progression and prognosis.

Key words Centromere protein H (CENP-H), breast cancer, prognosis, biomarker, proliferation

   Breast cancer is the most common cancer in females, and its incidence is increasing. The mean 5-year relapse-free survival rate of breast cancer is approximately 60%, but this value differs significantly across individuals[1]. Gene expression profiles have been reported to play an important role in predicting cancer progression or prognosis compared to traditional

Authors' Affiliations: [1]State Key Laboratory of Oncology in South China, Guangzhou, Guangdong 510060, P. R. China; [2]Department of Experimental Research, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong 510060, P. R. China; [3]Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangdong Provincial Key Laboratory of Molecular Tumor Pathology, Guangzhou, Guangdong 510515, P. R. China.

Corresponding Author: Li-Bing Song, Room 633, Cancer Center, Sun Yat-sen University, 651 Dongfeng Road East, Guangzhou, Guangdong 510060, P. R. China; Tel: +86-20-87343186; Fax: +86-20-87335828; Email: LB.Song1@gmail.com.

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parameters such as lymph node status and tumor grade, among others[2,3]. However, few prognostic markers have practical value. Therefore, to find reliable breast cancer molecular markers that can accurately predict outcomes for patients remains a great challenge.

   Chromosomal instability, a state of frequent chromosome loss and gain during cell division[4], is a hallmark of human cancers. Like many other malignancies, most breast cancers have significantly abnormal genomic structure, including aberrant numbers of chromosomes. In breast cancer cell lines, both variable and unstable chromosome numbers were observed[5]. Reports have also shown that aneuploidy is a prognostic factor in breast cancer[6]. The gain of chromosome 20 is prevalent in patients with breast carcinoma and may serve as a valuable prognostic marker[7]. Additionally, structural genomic aberrations, including aneuploidy, frequently occur in breast cancer[8-10]. Thus, chromosomal instability and aneuploidy may be associated with breast cancer development and

progression.

Centromere protein H (CENP-H), a component of the kinetochore that is active in centromeres of stable dicentric chromosomes [11], plays an essential role in appropriate kinetochore assembly as well as accurate chromosome segregation [12-14]. CENP-H is considered to be crucial for cell growth and mitotic progression[14]. It is known to be up-regulated in colorectal cancers [15-20]. Moreover, ectopic CENP-H expression in a diploid colorectal cancer cell line can induce chromosome missegregation and aneuploidy [15]. CENP-H is also deregulated in oral squamous cell carcinomas. Its expression in oral squamous cell carcinomas is significantly correlated with cell proliferation in malignant conditions [16]. Previously, we found that CENP-H was deregulated in several epithelium-originated carcinomas, such as esophageal carcinoma [17], nasopharyngeal carcinoma[18], non–small cell lung cancer[19], and tongue cancer[17-20]. We also provided evidence for a direct role of CENP-H in chromosome instability and carcinogenesis. CENP-H expression may be a valuable prognostic marker to predict the early stages of epithelium -originated cancers. However, it is unknown whether deregulation of CENP-H is involved in breast cancer development and progression.

In this study, we aimed to investigate CENP-H expression in cell lines simulating different stages of breast cancer development and progression. More specifically, we used immunohistochemistry to investigate CENP-H expression in 307 samples of breast cancer to determine the correlation of CENP-H expression with clinicopathologic characteristics, to evaluate the role of CENP-H in breast cancer progression, and to determine its prognostic value.

Materials and Methods

Patients and tissue specimens

The present study was conducted on 307 samples of paraffin-embedded breast cancer and 30 samples of adjacent non-cancerous tissues, which were histologically and clinically diagnosed at the Sun Yat-sen University Cancer Center between March 1, 1999 and December 25, 2002. The clinical information of these samples is described in detail in Table 1. Conventional clinical features including age, clinical stage, T classification, N classification, distant metastasis, estrogen receptor, C-ErbB-2, and progesterone receptor were all available. All patients were females with an average age of 48.9 years (range, 26 to 80 years). A total of 262 patients were followed up at 6-month

intervals for the first 5 years after the diagnosis and then annually in an outpatient clinic. The median follow-up time was 58 months (range, 4 to 78 months). Survival time was calculated from the date of operation to the time of death or the most recent follow-up if the patient was alive. This study was approved by an institutional ethical committee.

Cell lines

A primary culture of the normal mammary epithelial cell line BN6 was established from a biopsy of non-cancerous mammary epithelium and was cultured in complete Keratinocyte-SFM (Invitrogen, Carlsbad, California, USA). Human immortalized mammary epithelial cell line MCF10A and breast cancer cell lines MCF7, MDA-MB-435S, and MDA231 were purchased from American Type Culture Collection (ATCC). MCF10A was cultured essentially as described [21] and grown in DMEM/F12 plus 5% horse serum. MCF7 and MDA-MB-435S cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). MDA231 cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen). The primary culture of mammary epithelial cells was initiated as described before [22]. Full-length CENP-H was cloned into pMSCV vector and retroviruses were generated as described [22]. The CENP-H gene was introduced into mammary epithelial cell lines by infection with retroviruses containing pMSCV-CENP-H for 48 h. Cells were selected and maintained in 0.5 μg/mL puromycin for 4 days. Western blot was performed to confirm the expression of CENP-H.

RNA extraction and real-time reverse transcription-polymerase chain reaction

Total RNA from cultured cell lines or frozen tissue biopsies were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For quantitative real-time RT-PCR analysis, 0.2 μg RNA from each sample was reversely transcribed with TaqMan reverse transcription reagents and random hexamer primers (Applied Biosystems, Inc., Foster City, Calif). The primers and probes for the real-time RT-PCR were designed with Primer Express version 2.0 (Applied Biosystems, Inc.) as described previously[19]. Expression data were normalized to the geometric mean of housekeeping gene *GAPDH* to control the variability in expression levels and were analyzed using the $2^{-\triangle\triangle CT}$ method.

Western blotting

Cultured cells were subjected to standard Western blot analysis as described previously [18]. A polyclonal rabbit antibody against CENP-H (1:500, Bethyl Laboratories, Montgomery, TX) was used to detect CENP-H protein expression in breast cancer cell lines as well as normal and immortalized mammary epithelial cell lines. Protein levels of anti–α-tubulin mouse monoclonal antibody (1:1000, Santa Cruz, Santa Cruz, CA) were examined by reprobing the same blot and served as loading control.

Immunohistochemistry

CENP-H expression was determined by immunohistochemistry on paraffin-embedded tissue samples as described earlier [18]. Briefly, 4-μm sections were cut from paraffin-embedded tissue blocks. Paraffin sections on the silane-coated slides were deparaffinized with xylene, rehydrated through a graded ethanol series, and underwent antigen retrieval by microwave boiling in EDTA antigenic retrieval buffer. Subsequently, sections were blocked with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. The sections were incubated with 1% BSA to block non-specific binding and were then incubated overnight at 4℃ with rabbit polyclonal anti-CENP-H (1:500, Bethyl Laboratories, Montgomery, TX) or mouse monoclonal anti-Ki-67 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber. For the negative control, normal goat serum was used to replace anti–CENP-H antibody. Counterstaining was performed using 10% Mayer's hematoxylin. The degree of CENP-H staining in the sections was observed and

Table 1. Clinicopathologic characteristics of patients with breast cancer and expression of centromere protein H (CENP-H) in breast cancer

Characteristic	No. of cases	Percentage (%)
Age (years)		
≤45	124	40.4
>45	183	59.6
Clinical stage		
I	35	11.4
II	146	47.7
III	90	29.1
IV	36	11.8
T classification		
T1	66	21.9
T2	157	52.0
T3	59	18.0
T4	25	8.1
N classification		
N0	128	41.8
N1	117	38.2
N2	49	15.7
N3	13	4.3
Distant metastasis		
Yes	14	4.6
No	293	95.4
Ki-67 expression		
Low	196	63.8
High	111	36.2
CENP-H expression		
Negative (–)	58	18.9
Positive (+~+++)	249	81.1
Low (–~+)	173	56.4
High (++~+++)	134	43.6
Patients with follow-up	262	85.3
Survival status (at follow-up)		
Alive	171	65.3
Death because of breast cancer	79	30.1
Death because of other causes	12	4.6

evaluated as described previously [18]. The proportion of CENP-H–expressing cells varied from 0 to 100%. The cells at each intensity of staining were recorded on a scale of 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown), and 3 (strong staining, brown). An optimal cutoff value was identified on the basis of a measure of heterogeneity with the log-rank test statistic with respect to overall survival. Tumors with an intensity score of ≥2 and at least 50% of malignant cells with positive CENP-H staining were classified with high expression (++ to +++), and others were classified with low expression (– to +) of CENP-H antigen.

Statistical analysis

The Mann-Whitney U test was used to analyze the CENP-H expression level and clinicopathologic parameters. The Kaplan-Meier method was use to plot survival curves, and the observed differences in survival were compared by the log-rank test. The Cox proportional hazards regression model in multivariate analysis was used to analyze the significance of various clinicopathologic parameters. A P value less than 0.05 was considered statistically significant.

Results

CENP-H expression is elevated in breast cancer

We examined CENP-H mRNA and protein expression in normal mammary epithelial cell line BN6, immortalized mammary epithelial cell line MCF10A, and breast cancer cell lines MCF7, MDA231, and MDA-MB-435S. MCF10A, MCF7, MDA231, and MDA-MB-435S cells showed higher CENP-H expression than BN6 cells (Figure 1A). To determine if CENP-H
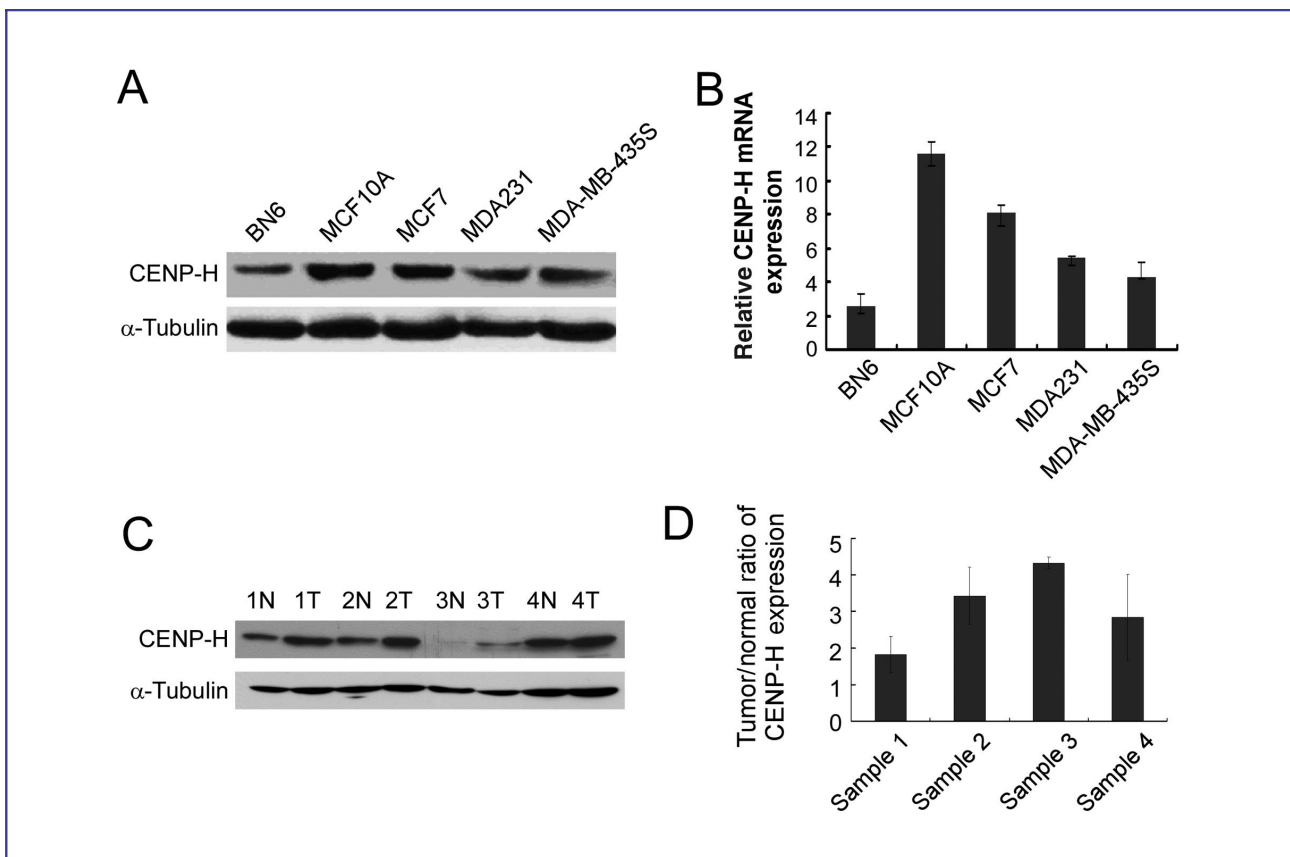

Figure 1. **CENP-H expression is elevated in breast cancer.** A, CENP-H expression in normal mammary epithelial cell line BN6, immortalized mammary epithelial cell line MCF10A, and breast cancer cell lines MCF7, MDA231, and MDA-MB-435S was analyzed by Western blot. B, relative expression level of CENP-H in BN6, MCF10A, MCF7, MDA231, and MDA-MB-435S cell lines quantified by real-time RT-PCR. *Columns*, means from 3 parallel experiments; *bars*, standard deviation (SD). C, CENP-H expression in paired primary breast tumors (T) and normal breast tissues (N) from the same patients detected by Western blot. D, average tumor/normal (T/N) ratios of CENP-H expression quantified by real-time RT-PCR. *Bars*, SD.

was also up-regulated at the mRNA level, real-time RT-PCR was performed. Concomitant with up-regulation of the CENP-H protein, there was 14-fold up-regulation of *CENP-H* mRNA in MCF10A, MCF7, MDA231, and MDA-MB-435S cells compared with that in BN6 cells (Figure 1B).

To verify whether CENP-H up-regulation is clinically correlated with breast cancer development and progression, comparative analysis of CENP-H expression was conducted on 4 cases of paired primary breast cancer tissues and their adjacent non-cancerous tissues. Western blot revealed that CENP-H expression was up-regulated in all 4 human primary breast cancer tissues compared with that in the matched adjacent non-cancerous tissues (Figure 1C). Real-time RT-PCR analysis showed that the tumor/normal (T/N) ratio of *CENP-H* mRNA expression was as high as 35-fold in one of the 4 paired primary breast cancer tissues (Figure 1D).

CENP-H expression in archived breast cancer tissues

Further, to investigate CENP-H expression in breast cancer tissues and to evaluate its clinicopathologic significance, 307 cases of paraffin-embedded, archived breast cancer tissues and 30 cases of adjacent normal tissues were examined by immunohistochemistry using an antibody against human CENP-H. Only 40.0% (12/30) of adjacent normal tissues showed weak positive signals (Figure 2A and 2B). On the other hand, 81.1% (249/307) of breast cancer tissues showed weak or strong CENP-H staining, which was mainly observed in tumor epithelial cells, not in surrounding stromal cells (Figure 2C-2F). In our previous study [23], CENP-H was found to be dominantly expressed in the nuclei. Notably, CENP-H was also detected in some areas of the hyperplasia of the mammary glands (Figure 2G and 2H).

The relationship of CENP-H up-regulation with the clinicopathologic parameters of breast cancer

The Mann-Whitney U test was used to evaluate the clinicopathologic significance of CENP-H expression in breast cancer. As shown in Table 2, CENP-H expression was related with clinical stage ($P = 0.001$), T classification ($P = 0.032$), and N classification ($P = 0.018$). Spearman correlation analysis revealed that CENP-H expression was positively correlated with clinical stage ($r = 0.192$, $P = 0.001$), T classification ($r = 0.123$, $P = 0.031$), and N classification ($r = 0.135$, $P = 0.018$). No significant correlation was found between CENP-H expression and age or distant metastasis.

We further investigated the relationship between CENP-H expression and breast cancer proliferation using immunohistochemistry to analyze Ki-67 expression. Areas with low CENP-H expression had asteroid detectable staining for Ki-67 (Figure 3A and 3B), whereas those with high CENP-H expression also had strong Ki-67 staining signals (Figure 3C and 3D). The Mann-Whitney U test revealed that Ki-67 expression was positively correlated with CENP-H expression in breast cancer ($r = 0.241$, $P < 0.001$) (Table 2).

Survival analysis

Kaplan–Meier univariate survival analysis was performed on the 262 breast cancer patients for whom follow-up data were available. A log-rank test showed that the survival time was significantly longer in the low CENP-H expression group than in the high expression group ($P = 0.001$) (Figure 4). The cumulative 5-year survival rate was 72.3% (95% CI, 65.1%–79.5%) in the low CENP-H protein expression group ($n = 148$), but it was only 51.5% (95% CI, 42.2%–60.8%) in the high expression group ($n = 114$). In addition to CENP-H expression, the impact of classical clinicopathologic characteristics (including clinical stage, T classification, N classification, and distant metastasis) on survival was analyzed, and these characteristics were all significantly correlated with survival in the Kaplan-Meier analysis and the log-rank test ($P < 0.001$). Therefore, multivariate survival analysis, including CENP-H expression level, clinical stage, T classification, N classification, and distant metastasis, was performed. This analysis indicated that T classification, N classification, distant metastasis, and CENP-H expression level were independent prognostic indicators of patient survival (Table 3).

Because CENP-H was a potential prognostic marker for patients with early stage cancers in our previous study[18-20], we also analyzed the significance of CENP-H expression in the subgroups stratified on the basis of tumor clinical stage. We found that patients with tumors exhibiting high CENP-H expression had significantly lower overall survival rates in the subgroups of clinical stage I–II ($P = 0.001$) (Figure 5A), whereas there was no significant difference in survival in the subgroup of clinical stage III–IV ($P = 0.38$) (Figure 5B). Similarly, patients with high CENP-H expression had poorer overall survival rates in the N0–1 subgroup ($P = 0.001$) (Figure 5C), but not in the N2–3 subgroup ($P = 0.65$) (Figure 5D). However, the expression levels of CENP-H are strongly correlated with patients' survival in both T1–2 and T3–4 subgroups (Figure 5E and 5F). These results suggest that CENP-H may be a valuable prognostic
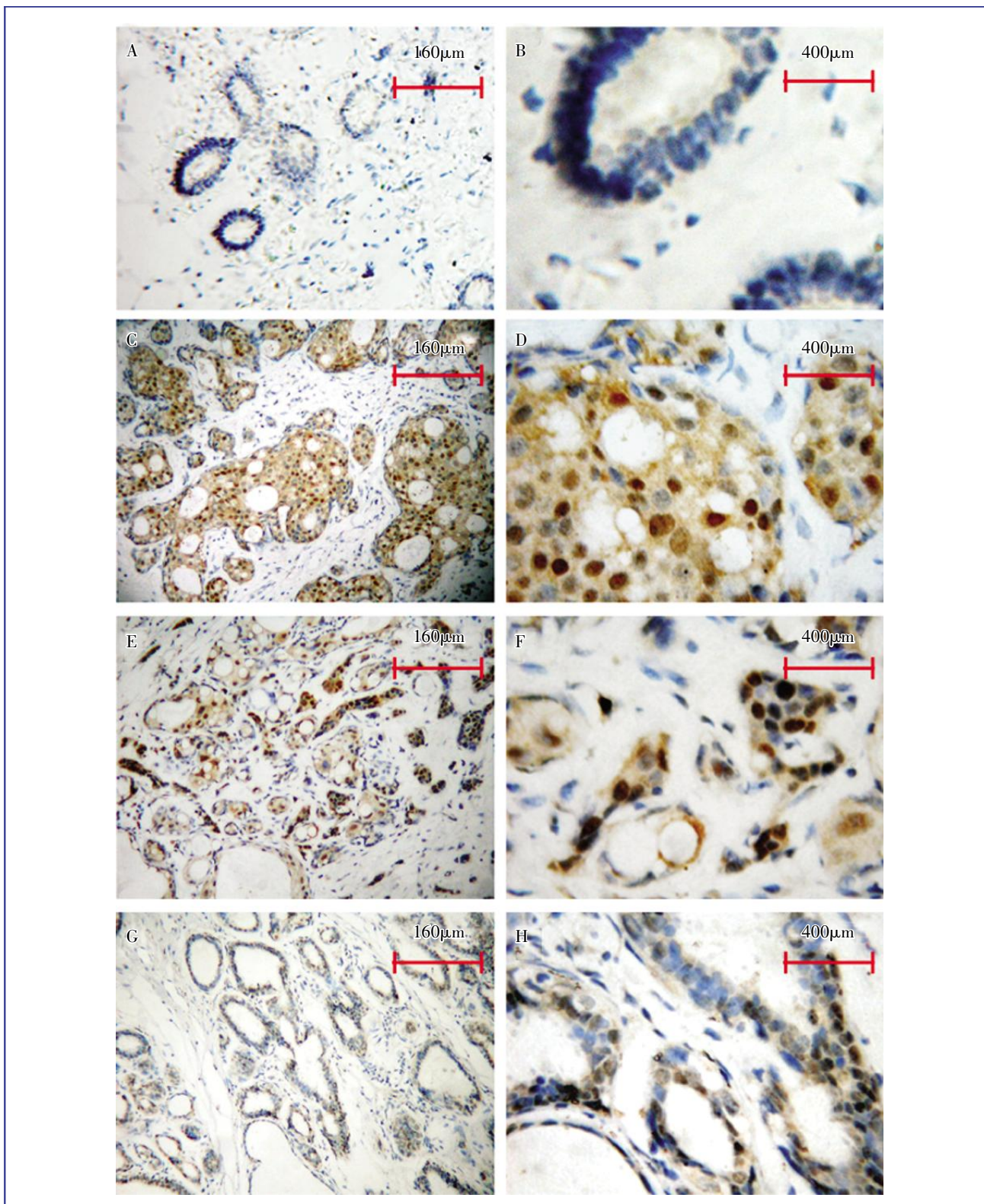
Figure 2. Representative images from immunohistochemical assays of breast cancer cases. CENP-H shows weak or undetectable staining in normal epithelial cells (A,B), strong nuclear or nuclear/cytoplasmic staining in breast cancer with low invasive ability (C,D) and in that with high invasive ability(E,F). CENP-H shows moderate nuclear staining in the hyperplasia areas of the mammary glands(G,H).
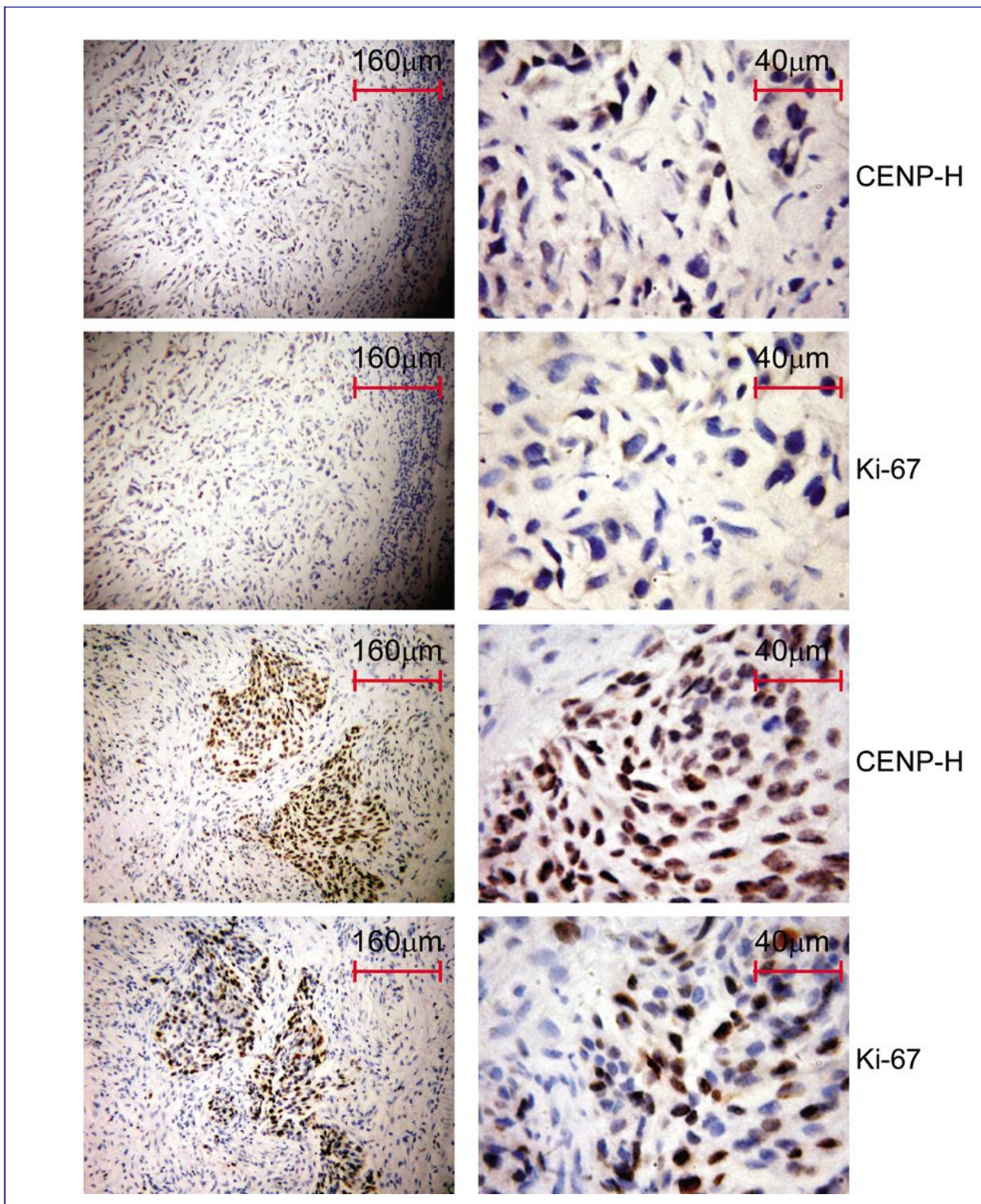
Figure 3. CENP-H expression in breast cancer tissues is positively correlated with Ki-67 expression. Immunohistochemical staining of CENP-H and Ki-67 in a series of sections from paraffin blocks of breast cancer reveals weak CENP-H expression, weak Ki-67 expression, strong CENP-H expression, and strong Ki-67 expression.

Table 2. Correlation of centromere protein H (CENP-H) expression to clinicopathologic characteristics of patients with breast cancer

Characteristic		CENP-H expression [cases (%)]		P	Spearman's r value
		Low	High		
Age (years)	≤45	69 (22.5)	55 (17.9)	0.508	−0.006
	>45	104 (33.9)	79 (25.8)		
Clinical stage	I	21 (39.4)	14 (4.6)	0.001	0.192
	II	96 (31.3)	50 (16.3)		
	III	44 (14.3)	46 (15.0)		
	IV	12 (3.9)	24 (7.8)		
T classification	T1	41 (13.4)	25 (8.1)	0.032	0.123
	T2	92 (30.0)	65 (21.2)		
	T3	33 (10.7)	26 (8.5)		
	T4	7 (2.3)	18 (5.9)		
N classification	N0	80 (26.1)	48 (15.6)	0.018	0.135
	N1	66 (21.5)	51 (16.6)		
	N2	22 (7.2)	27 (8.8)		
	N3	5 (1.6)	8 (2.2)		
Distant metastasis	No	168 (54.7)	125 (40.7)	0.112	0.091
	Yes	5 (1.6)	9 (2.9)		
Ki-67 expression	Low	121 (39.4)	75 (24.4)	<0.001	0.241
	High	40 (13.1)	71 (23.1)		

Table 3. Cox univariate and multivariate analysis of different prognostic parameters in patients with breast cancer

Variate	No. of patients	Univariate analysis		Multivariate analysis		
		P	Regression coefficient (SE)	P	Relative risk	95% confidence interval
T classification		<0.001	0.598 (0.125)	0.001	1.193	1.193–1.969
T1–T2	190					
T3–T4	72					
N classification		<0.001	0.652 (0.110)	<0.001	1.354	1.354–2.143
N0–N1	208					
N2–N3	54					
Distant metastasis		<0.001	1.773 (0.327)	<0.001	4.224	2.167–8.235
No	253					
Yes	9					
CENP–H expression		0.002	0.668 (0.212)	0.023	1.635	1.070–2.498
Low	148					
High	114					

marker for early-stage breast cancer patients.

Discussion

In this study, we investigated the clinicopathologic significance and prognostic value of CENP-H in breast cancer. By using RT-PCR and Western blot, we found that CENP-H was overexpressed in immortalized mammary epithelial cell lines and breast cancer cell lines, but not in normal mammary epithelial cells. Additionally, the expression of *CENP-H* mRNA was higher in breast cancer biopsies than in adjacent normal tissues. These findings suggested that CENP-H was up-regulated in breast cancer cells and breast cancer tissues at both the transcriptional and translational levels. These results are similar to the findings of a previous study suggesting that CENP-H is up-regulated in colorectal cancer[15].

To explore the clinicopathologic significance and prognostic value of CENP-H in breast cancer, we examined CENP-H expression in 307 breast cancers and 30 adjacent normal tissues using
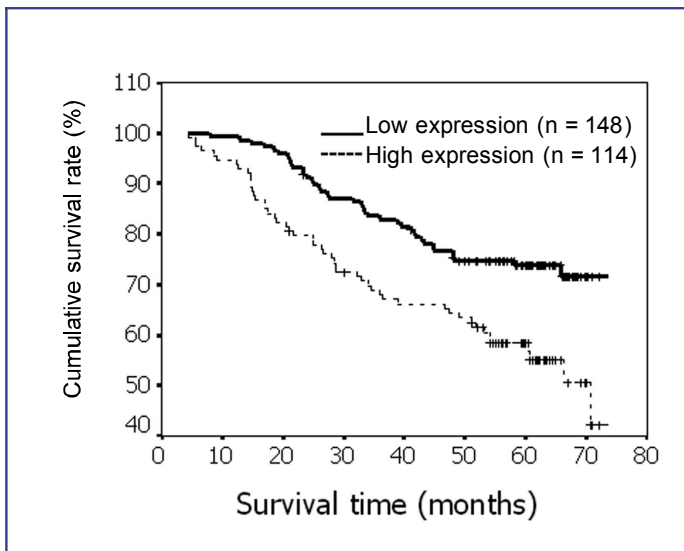
Figure 4. **Kaplan-Meier survival curves for breast cancer patients with low CENP-H expression (bold line) versus high CENP-H expression (dotted line).** The difference was compared by the log-rank test. The survival rate was significantly higher in the high expression group than in the low expression group (P = 0.0012).

immunohistochemistry and compared these findings with clinicopathologic parameters. Over 80% of breast cancers showed weak or strong CENP-H expression, whereas only 40% of adjacent normal tissues showed weak CENP-H staining. Statistical analysis showed that CENP-H expression level was positively correlated with clinical stage, T classification, and N classification, suggesting that CENP-H may play an important role in the development and progression of breast cancer and may be a new marker to identify patients with aggressive breast cancers.

This study also showed that CENP-H was overexpressed in the hyperplasia areas of the mammary epithelium during the early stages of breast cancer and hence was an indicator of the early stage of cancer development. Additionally, high CENP-H expression primarily indicated poor survival in early-stage breast cancer patients. Compared with the expression in normal epithelial cells, CENP-H expression level is much higher in immortalized cell lines, which represent the early stage of carcinogenesis; hence, we propose that CENP-H may play an important role in the early stage of breast cancer development. CENP-H is essential for cell growth and mitotic progression [14]. Since our previous data indicated that CENP-H may be a marker of cancer development [19,20], our goal was to analyze the relationship between CENP-H and Ki-67, one of the most frequently used markers of proliferating cells. We found a significant correlation between the gene expression of CENP-H and that of Ki-67, indicating that CENP-H is associated with cell proliferation *in vivo*. This finding provides more evidence that CENP-H may play an important role in the early stage of tumorigenesis. We

have previously published similar results, showing that CENP-H may be an early marker of nasopharyngeal carcinoma [18]. These results suggest a crucial role for CENP-H in the early development of epithelium-originated cancers and provide evidence that CENP-H can be used as a potential molecular marker for early cancer diagnosis and treatment. However, the other functions and mechanisms underlying CENP-H action remain to be elucidated.

Early stage breast cancer has a highly variable prognostic status and benefits to individual patients from available treatments are critically unpredictable. Although traditional prognostic factors such as lymph node status, tumor grade, histological grade, and distant metastasis are helpful, attention must be paid to the molecular prognostic markers to help identify low-risk from high-risk patients. Recently, CENP-F, another kinetochore component protein, was reported to be associated with poor prognosis and chromosomal instability in patients with primary breast cancer [24]. This suggests that kinetochore proteins can be used to predict the prognosis of patients suffering from breast cancer. In this study, we showed the prognostic value of CENP-H expression in breast cancer patients. Higher CENP-H protein expression was correlated with reduced overall survival in both univariate and multivariate analyses, highlighting the value of CENP-H as a marker of poor prognosis for breast cancer. CENP-H has a particularly great value in predicting the prognosis of early-stage breast cancer patients. These findings are also in agreement with our previous study, where we analyzed CENP-H expression and overall survival in patients with nasopharyngeal carcinoma [18]. Our findings not only
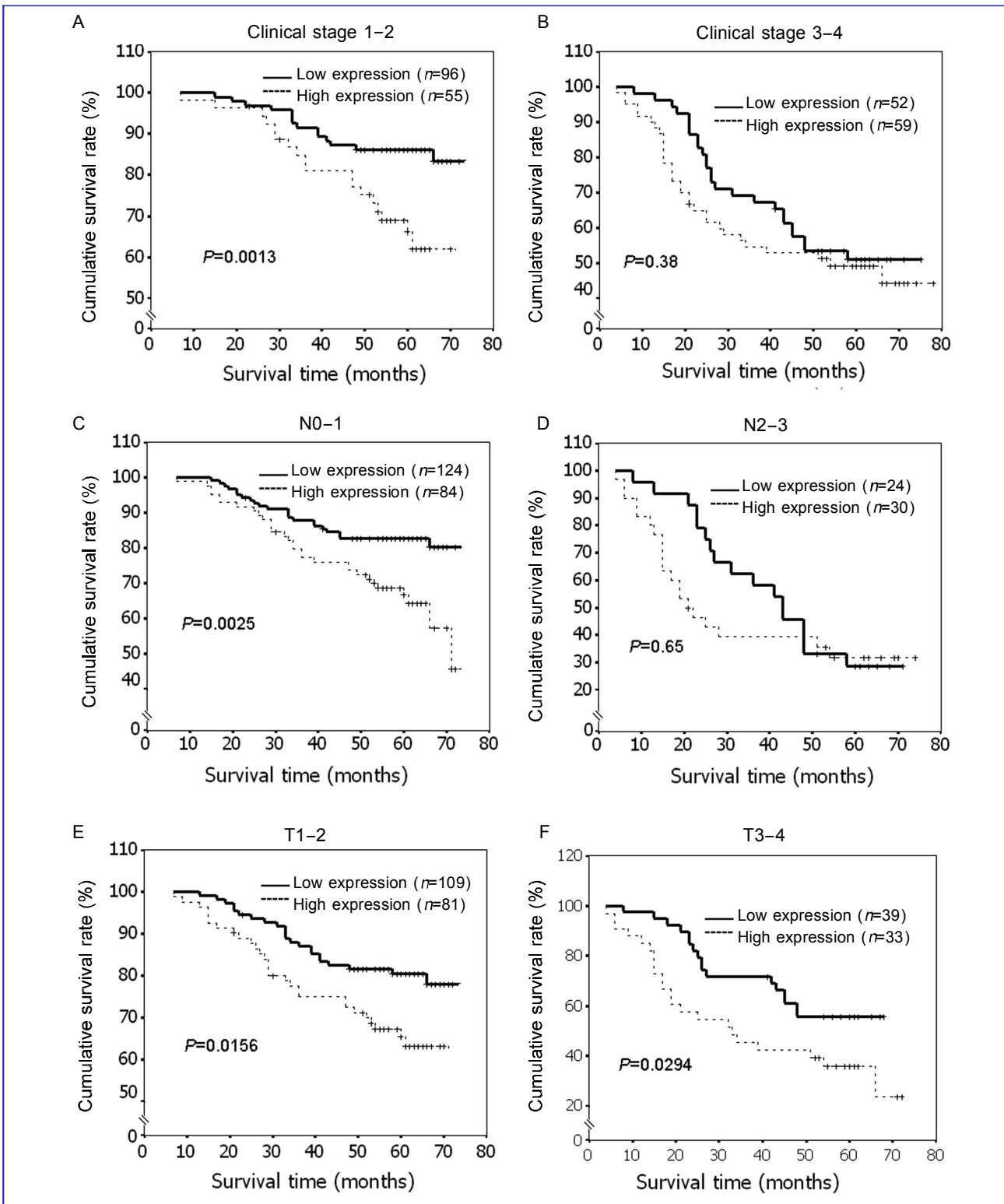
Figure 5. Kaplan-Meier analysis showing the overall survival of patients categorized according to different stages/classifications and status of CENP-H expression. The curves of patients with high and low expression of CENP-H were compared in the following patient subgroups: stage I-II and stage III-IV (A, B), T classification, N0–N1 and N2–N3 (C, D) and N classification T0–T2 and T3–4 (E, F). In subgroups of stage 1–2, N0–1, T1–2 and T3–4, the survival rates were significantly higher in patients with low CENP-H expression than in those with high CENP-H expression ($P < 0.05$).

indicate the prognostic significance of CENP-H in these cancers but also suggest a potential role for CENP-H in the early stage of cancer development.

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