Centromere Protein I (CENP-I) Is Upregulated in Gastric Cancer, Predicts Poor Prognosis, and Promotes Tumor Cell Proliferation and Migration

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Abstract
This study aimed to investigate the expression and cellular function of the centromeric family of proteins (CENPs), especially centromere protein I (CENP-I), in gastric cancer (GC) and identified its clinical significance and cellular functions. CENP-I expression in GC was studied by cDNA microarray, quantitative real-time PCR (qRT-PCR), and immunohistochemistry (IHC), and using datasets from The Cancer Genome Atlas (TCGA), UALCAN, and Gene Expression Omnibus (GEO) databases. Microarray and bioinformatic analyses identified upregulated CENP-A/E/F/H/K/P/W and HJURP in stomach adenocarcinoma (STAD), but not in signet ring cell carcinoma (SRCC). Significantly higher CENP-I mRNA expression was also confirmed in 40 pairs of GC tissues than in paired normal gastric tissues by qRT-PCR ($P < .001$). IHC showed that elevated CENP-I expression was associated with higher tumor stage, lymph node invasion, increased HER2-positive rate (36.7% vs 10.0%), and intestinal Lauren classification in 69 GC samples compared to paired paracancerous normal tissues. The survival of the high-CENP-I group members was poor compared with that of the low-CENP-I group ($P = .0011$). Cox univariate regression analysis identified tumor size ($P = .008$), HER2 status ($P = .027$), and CENP-I expression ($P = .049$) were independent prognostic factors of GC. The cellular function of CENP-I was studied in MKN45 and MKN28 GC cell lines in vitro. Cell proliferation, migration, and apoptosis were determined using CCK-8, transwell assay, TUNEL assay, and flow cytometry. Our results showed that CENP-I promoted GC cell proliferation, inhibited apoptosis, facilitated cell migration, and induced epithelial-mesenchymal transition (EMT), possibly by activating the AKT pathway. CENP-I expression was correlated with genetic signatures of the proliferative subtype of GC, characterized by intestinal Lauren classification, HER2 amplification, and TP53 mutation. In conclusion, this study revealed an elevated CENP-I expression in GC, which was associated with malignant features and poor prognosis of GC patients, and identified its function in modulating cell proliferation, apoptosis, and migration.

Keywords
gastric cancer, mitotic checkpoints, centromere protein I, Lauren classification, proliferative subtype

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Abbreviations
CCK-8, cell counting kit-8; CIN, chromosomal instability; CENP, centromere protein; CRC, colorectal cancer; DMEM, Dulbecco’s modified eagle’s medium; ECL, electrochemiluminescence; EGFP, enhanced green fluorescent protein; EMT, epithelial–mesenchymal transition; ER+, estrogen receptor-positive; FBS, fetal bovine serum; GC, gastric cancer; GEO, Gene Expression Omnibus; HRP, horseradish peroxidase; IHC, immunohistochemistry; OD, optical density; qRT-PCR, quantitative real-time PCR; RIPAI, radio-immunoprecipitation assay; RPMI, Roswell Park Memorial Institute; SRCC, signet ring cell carcinoma; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling

Introduction
Gastric cancer (GC) is the fifth most frequently diagnosed cancer type and the third leading cause of cancer-related death, with over 1 million new diagnoses and more than 780,000 deaths (8.2% of all cancer deaths) each year.1,2 GC falls into 6 major histological subtypes according to the WHO classification in 2000: stomach adenocarcinoma (STAD, including tubular adenocarcinoma, papillary adenocarcinoma, and mucinous adenocarcinoma), signet ring cell carcinoma (SRCC, also called mucinous carcinoma), squamous cell carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, and carcinoid, and other rare subtypes.3 The etiology of GC is multifactorial, with complex host genetic and environmental factors contributing to its development. Thus, it is of great importance for the prevention and treatment of GC to improve our understanding of the molecular basis of the malignancy.

Mitotic checkpoint regulation is essential for ensuring accurate chromosome segregation during mitosis, and its dysregulation promotes tumorigenesis in vitro and in vivo.4 Mitosis requires the recruitment of a large proteinaceous structure termed the kinetochore onto chromosomal centromeric DNA, which involves the assembly of a constitutive centromere-associated network of 16 proteins that are distributed in several functional groups, including the CENP-C, CENP-H1/K complex, CENP-L/M/N complex, CENP-O/P/Q/R(U(50)) complex, CENP-T/W, and CENP-S/X, onto the centromeric CENP-A chromatin, mediated by the CENP-A chaperone HJURP.5 Considering the importance of centromere proteins (CENPs) in mitosis and their likely involvement in tumorigenesis, this study focused on the investigation of the role of centromeric proteins, specifically centromere protein I (CENP-I), in GC.

Materials and Methods
Ethics Statement
Ethical approval to report this case series was obtained from the institutional ethics committee on December 31, 2018 (IRB No.: YHD 2018-150). Written informed consent was obtained from patients for the use of clinical tissue samples and their anonymized information published in this article.

Molecular Cloning
A CENP-I overexpression plasmid was constructed by inserting the 2271 bp CENP-I coding sequence (GenBank accession No.: NM_006733.2) into the pCMV6-Entry vector. For CENP-I knockdown, 3 CENP-I-specific shRNA (target sequences: shCENP-I-1: gcTCTTTTATCAGTACCAT, shCENP-I-2: AGGCTTTGTGTCACTGTATA, shCENP-I-3: TTGCAA ATGGCAGTGAGAT) were expressed from the GV102 vector [Genchem, China]. Non-targeting control shRNA [shNC: TTCTCCGAACGTGTCACGT] was used as a negative control.

RNA Extraction and Quantitative Real-Time PCR
Pathologically confirmed specimens were collected during surgery from patients admitted to our institution, kept in liquid nitrogen within 30 min, and then transferred for storage at −80°C until RNA extraction. Paracancerous tissues were collected 2 cm from the margin where the tissue was negative after pathological examination. Total RNA was extracted from tissues or cells using the TRIzol™ Plus RNA Purification Kit (Invitrogen, USA) according to the manufacturer’s instructions. Total RNA was reverse transcribed using the PrimeScript II first Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) analysis was routinely performed using β-tubulin as an endogenous reference. The mRNA expression levels of target genes were compared using the 2−ΔΔCt method. All experiments were performed in triplicate. The primer sequences were as follows: CENP-I-F, 5′-CAGATGGGCTCTCAGT GCTAAA-3′, CENP-I-R: 5′-ATGGATCCAGAAGTTGG TAAA-3′, β-Tubulin-F: 5′-GGACCGATCTCTGTGACTA-3′, β-Tubulin-R: 5′-CTTTGGCCAGTGTTACCT-3′.

Western Blotting
Total protein was extracted from cells lysed with radio-immunoprecipitation assay (RIPA) Lysis Buffer (Beyotime, China) and subjected to western blotting using rabbit or mouse primary antibodies from Abcam, USA: anti-CENPI (ab118796, 1:1000 dilution); Affinity Biosciences, USA: anti-GAPDH (T0004, 1:2000 dilution), anti-β-actin (T0022, 1:2000 dilution), anti-β-tubulin (T0023, 1:2000 dilution), phospho-pan-AKT1/2/3 (Thr308) (AF3262, 1:1000 dilution), pan-AKT1/2/3-C-terminal (AF0791, 1:1000 dilution); or Cell Signaling Technology, USA: epithelial–mesenchymal transition (EMT) Antibody Sampler Kit (9782T, 1:1000 dilution).
Table 1. Fold Change of the Expression of Centromeric Proteins in Stomach Adenocarcinoma and Signet Ring Cell Carcinoma Against Para-Cancerous Normal Tissues by Microarray.

| Gene symbol | Probe name | Probe sequence | Genbank accession | Fold change (STAD vs normal) | Fold change (SRCC vs normal) |
|-------------|------------|----------------|-------------------|-------------------------------|-------------------------------|
| CENP-A      | A_24_P413884 | TAGTTTGTGAGTTACTCATGTGACTATTTGAGATTGAAAAACATCGATTTTCTGGGG | NM_001809 | 3.5259418 | 1.5203211 |
| CENP-E      | A_23_P253524 | CTGAGTGCAAAACTCGAGCTCCTCTCTCTTGGACATTCAGATCCTCTTAA | NM_001813 | 2.9304266 | 1.1442649 |
| CENP-F      | A_24_P96780 | GCTGGAGATAGACCTTTTAAAGTCTAGTAAAGAAGCTCAATAATTCATTGAAAGCTAC | NM_016343 | 3.9159503 | 1.1696385 |
| CENP-H      | A_23_P110802 | TTACATTCTGCAACCTTTATTTATTTGGAATACATCTCTGTGACATTCTGTCGAC | NM_022909 | 2.822752 | 1.091639 |
| CENP-I      | A_33_P3221313 | TCCCAGAGCAGGAGGGCATAAACTGCAACAATCAAATATTTGCATGACATAACACTG | NM_006733 | 5.23768 | −1.081777 |
| CENP-K      | A_23_P155989 | TTGAGGTAATATCTGAGAGTGACATTTAAATATTTATAGCAATTTGTTTCATATATA | NM_022145 | 2.5990665 | −1.0310805 |
| CENP-P      | A_33_P3245321 | TGGAAATCGAAGCTGCTCTGGAAAGCCTGAAAATCGCTTTGTGGCAGGAAGAACAACAGA | NM_001012267 | 2.2836795 | 1.7405572 |
| CENP-W      | A_24_P462899 | GAGAGCAGAGGTTAGAAGTCGAAGAAACATATTTGGAAGTATGATGGCATTCTTTG | NM_001012507 | 2.4428961 | 1.603932 |
| HJURP       | A_33_P3807062 | CATCAGAGATAACCTGAGTTCTGTGAGAATTTATGTAATAAAGTGCTCAATTAG | NM_018410 | 4.137667 | −1.3971884 |

Abbreviations: STAD, stomach adenocarcinoma; SRCC, signet ring cell carcinoma.
and goat anti-rabbit/mouse horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-BIO, China, ZB-2305/ ZB-2301, 1:2000 dilution). Immunoblots were developed using the electrochemiluminescence (ECL) reagent (Thermo Fisher Scientific, USA). For quantification, densitometric analysis was performed by measuring the grayscale intensity of the target bands derived from scanned films using ImageJ software (imagej.nih.gov/ij).

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed as previously described using HRP-conjugated secondary antibody (ZSGB-BIO) for staining and DAB Kit (ZSGB-BIO) for color development. The slides were scored based on the intensity of the staining and the percentage of cells stained. Slides were visualized at ×200 magnification for scoring the staining intensity: no color for 0 points; light yellow for 1 point; yellow for 2 points; brown for 3 points. For each slide, 5 high magnification (×400) field were randomly selected to count positive cells ratio: less than 10% for 0 point; 10% to 25% for 1 point; 25% to 50% for 2 points; greater than 50% for 3 points. The 2 scores were added up as final score: 0 to 3 points were considered low CENP-I expression and 4 to 6 points were considered high CENP-I expression.

**Cell Culture and Transfection**

GC cell lines were purchased from iCell Bioscience (Shanghai, China). Cells were routinely cultured in Dulbecco’s Eagle’s medium (DMEM) (iCell Bioscience, Shanghai, China) or Roswell Park Memorial Institute (RPMI)-1640 medium (Biological Industries, Israel) supplemented with 10% fetal bovine serum (FBS, Biological Industries) at 37°C in humidified air containing 5% CO2 according to standard procedures. Transient transfection of plasmids into cells was achieved using XtremeGENE HP DNA Transfection Reagent (Roche, Switzerland) following the manufacturer’s instructions.

**Cell Counting Kit-8 Assay**

Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo Laboratories, Japan). For each well of a 96-well plate, 100 µL of cell suspension (2 × 10³-1 × 10⁴ cells) was inoculated. At each time point, the culture medium was replaced with 10 µL of CCK-8 solution plus 90 µL of growth media. Following 1 h incubation at 37 °C, the optical density (OD) at 450 nm was measured and plotted as growth curves.

**Transwell Migration Assay**

Transwell migration assays were performed as previously described with 4 × 10⁴ cells seeded into the upper chamber.

**TUNEL**

To assess cell apoptosis, 1 × 10⁵ cells/well were seeded into a 24-well plate. Where required, cisplatin (Beyotime) was added to the media 24 h before the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Two days after seeding, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% TritonX-100/PBS (Thermo Fisher Scientific) for 5 min, and processed using the One-Step TUNEL Apoptosis Assay Kit (Beyotime) following the manufacturer’s instructions. Cell nuclei were stained with Hoechst33358 (Beyotime) for 5 min. For each well, 10 random fields were captured using a fluorescence microscope. Percentage of apoptotic cells = (No. of EGFP [enhanced green

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**Table 2. Comparison of the Expression of Centromeric Proteins Between 415 Primary Stomach Adenocarcinoma and 34 Normal The Cancer Genome Atlas Samples as Analyzed by UALCAN Database.**

| Gene name | Median (Q1,Q3) (transcript per million) | Fold change | P value of expression | P value of effect on survival |
|-----------|----------------------------------------|-------------|-----------------------|-------------------------------|
| STAD      | Normal                                 |             |                       |                               |
| CENP-A    | 11.347 (7.068, 16.245)                 | 2.783 (0.275, 3.947) | 4.08                 | 1.62E-12                      |
| CENP-B    | 72.774 (53.084, 97.73)                 | 66.13 (2.188, 77.384) | 1.10                 | 8.04E-04                      |
| CENP-E    | 9.498 (5.534, 14.774)                  | 0.821 (0.505, 1.025) | 11.57                | 1.62E-12                      |
| CENP-F    | 14.511 (7.933, 23.092)                 | 1.418 (0.378, 2.043) | 10.23                | <1E-12                        |
| CENP-H    | 9.399 (6.161, 13.107)                  | 3.299 (2.481, 4.298) | 2.85                 | <1E-12                        |
| CENP-I    | 2.722 (1.496, 4.508)                   | 0.414 (0.202, 0.548) | 6.57                 | <1E-12                        |
| CENP-K    | 8.139 (5.354, 11.034)                  | 1.87 (0.437, 2.887)  | 4.35                 | <1E-12                        |
| CENP-L    | 8.215 (5.88, 10.786)                   | 2.391 (2.088, 2.877) | 3.44                 | 1.62E-12                      |
| CENP-M    | 21.483 (13.304, 31.521)                | 10.462 (1.023, 15.016) | 2.05                | 9.29E-12                      |
| CENP-N    | 14.985 (9.885, 19.7)                   | 5.299 (4.142, 6.411) | 2.83                 | <1E-12                        |
| CENP-O    | 9.959 (6.384, 3.66)                    | 2.981 (1.043, 4.053) | 3.34                 | 1.62E-12                      |
| CENP-P    | 3.289 (2.455, 4.354)                   | 1.41 (1.056, 1.906)  | 2.33                 | <1E-12                        |
| CENP-Q    | 5.079 (3.687, 7.101)                   | 2.297 (1.444, 2.993) | 2.21                 | <1E-12                        |
| CENP-T    | 14.816 (12.135, 17.988)                | 13.184 (8.701, 15.39) | 1.12                | 7.86E-03                      |
| CENP-W    | 38.184 (25.445, 55.522)                | 11.568 (6.849, 17.064) | 3.3                | 1.62E-12                      |
| HJURP     | 10.719 (6.839, 16.246)                 | 1.853 (0.245, 2.602)  | 5.78                 | 1.62E-12                      |

Abbreviations: STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas.


fluorescent protein]-expressing cells with red stain)/(total No. of EGFP-expressing cells) × 100%.

**Flow Cytometry**

Cell cycle status and apoptosis were detected by flow cytometry using the Cyteletest™ Plus DNA Reagent Kit (BD Biosciences, USA) and FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions.

**Bioinformatic Analysis**

Clinical data and RNA seq v.2 data were retrieved and analyzed using cBioPortal (www.cbioportal.org/), which was based on The Cancer Genome Atlas (TCGA) (cancergenome.nih.gov/) and Gene Expression Omnibus (GEO) databases (www.ncbi.nlm.nih.gov/geo/). CENP-I transcription levels and promoter methylation levels in STAD tissues were compared using the UALCAN (ualcan.path.uab.edu/) database.8

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software (v.8.0). Kaplan–Meier survival curves were compared using the log-rank (Mantel–Cox) test. Numeric data in accordance with normal distribution were compared using the Student’s t-test or one-way ANOVA. Categorical data were compared using the Fisher’s exact test. Univariate and multivariate regression analyses were performed using SPSS v.24.0.

**Results**

**Microarray and Bioinformatic Analysis Identified UpRegulation of Centromeric Proteins in Gastric Cancer**

To study the expression of centromeric proteins in GC, 2 pairs of STAD and SRCC tissues together with paired adjacent noncancerous tissues were subjected to cDNA microarray analysis. The results showed significantly elevated expression (fold change >2) of CENP-A, CENP-E, CENP-F, CENP-H, CENP-I, CENP-K, CENP-P, CENP-W, and HJURP in...
CENP-I was chosen as the focus of subsequent studies. We validated CENP-I mRNA expression levels in 40 pairs of GC and normal gastric tissues by qRT-PCR, which revealed higher expression of CENP-I in GC tissues than in normal gastric tissues (P<.001) (Figure 1B). In addition, CENP-I protein expression was also studied in 69 pairs of paraffin-embedded GC tissues and paracancerous normal tissues using IHC (Figure 1C). Correlations between CENP-I expression and patient clinicopathological characteristics were analyzed. We found significantly higher CENP-I expression in patients with higher tumor stage, lymph node invasion, HER2 overexpression (36.7% in high CENP-I samples vs 10.0% in low CENP-I samples), and intestinal Lauren subtype (Table 3). Pearson’s correlation analysis indicated that high CENP-I expression was correlated with advanced tumor stage (r²= .319, P= .008), HER2 overexpression (r²= .449, P<.001), and intestinal Lauren subtype (r²= .339, P= .004). Kaplan–Meier survival analysis showed significantly worse survival in patients with high levels of CENP-I (P= .0011) (Figure 1D), larger tumor size (P= .001), lymph node invasion (P= .024), higher tumor staging (P<.001), intestinal Lauren subtype (P= .032), and HER2 overexpression (P<.001). Univariate regression analysis of clinicopathological features over survival time was performed, followed by multivariate analysis of features in univariate analysis (P<.05) (Table 4). Results showed that tumor size (P= .008), HER2 status (P= .027), and CENP-I expression (P= .049) were independent prognostic factors for GC.

**Table 3. Correlations Between Centromere Protein I Protein Expression and Clinicopathological Characteristics in Gastric Cancer (GC).**

| Characteristics          | High CENP-I expression N (%) | Low CENP-I expression N (%) | P value |
|--------------------------|------------------------------|-----------------------------|---------|
| Age                      |                              |                             | >.999   |
| ≤60                      | 28 20 (29.0)                 | 8 (11.6)                    |         |
| >60                      | 41 29 (42.0)                 | 12 (17.4)                   |         |
| Gender                   |                              |                             |         |
| Female                   | 23 15 (21.7)                 | 8 (11.6)                    | .575    |
| Male                     | 46 34 (49.3)                 | 12 (17.4)                   |         |
| Clinical stage           |                              |                             |         |
| I to II                  | 30 17 (24.6)                 | 13 (18.9)                   | .0319*  |
| III to -IV               | 39 32 (46.4)                 | 7 (10.1)                    |         |
| Tumor grade              |                              |                             |         |
| G1 to G2                 | 44 34 (49.3)                 | 10 (14.5)                   | .170    |
| G3 to G4                 | 25 15 (21.7)                 | 10 (14.5)                   |         |
| Tumor depth              |                              |                             |         |
| T1 to T2                 | 16 11 (15.9)                 | 6 (8.7)                     | .547    |
| T3 to T4                 | 53 38 (55.1)                 | 14 (20.3)                   |         |
| Lymph node invasion      |                              |                             |         |
| Yes                      | 45 36 (52.2)                 | 9 (13.0)                    | .430    |
| No                       | 24 13 (18.8)                 | 11 (16.0)                   |         |
| Tumor size (cm)          |                              |                             |         |
| 1 to 4.5                 | 32 21 (30.4)                 | 11 (16.0)                   | .0392*  |
| >4.5                     | 37 28 (40.6)                 | 9 (13.0)                    |         |
| HER2                     |                              |                             |         |
| –                        | 49 31 (44.9)                 | 18 (26.1)                   |         |
| +                        | 20 18 (26.1)                 | 2 (2.9)                     |         |
| Lauren subtype           |                              |                             |         |
| Intestinal               | 43 35 (50.7)                 | 7 (10.1)                    | .0069** |
| Diffuse                  | 26 14 (20.3)                 | 13 (18.9)                   |         |

Abbreviation: CENP-I, centromere protein I. *: P<.05, **: P<.01.

STAD, among which CENP-I had the largest fold change of 5.24 times in terms of upregulation (Table 1). However, no statistically significant alteration in the expression of these genes was observed in SRCC.

The expression of 16 centromeric proteins in STAD tissues and patient survival were analyzed using TCGA RNA-seq data from UALCAN. The results showed significantly higher transcription levels of all 16 protein-encoding genes in primary GC tissues (n=415) than in normal tissues (n=34) (P<.0001) (Table 2). No correlation was found involving any of these proteins and patient survival.

From the above data, CENP-I was the centromere protein expressed in least abundance and was one of the most upregulated centromeric proteins in STAD (Figure 1A). Therefore, CENP-I was chosen as the focus of subsequent studies.

**Elevated Centromere Protein I Expression Associated with Malignant Status and Poor Gastric Cancer Patient Survival**

We validated CENP-I mRNA expression levels in 40 pairs of GC and normal gastric tissues by qRT-PCR, which revealed significantly higher CENP-I expression in least abundance and was one of the most upregulated centromeric proteins in STAD (Figure 1A). Therefore, CENP-I was chosen as the focus of subsequent studies.

To study the cellular function of CENP-I in GC, we measured the mRNA and protein abundance of CENP-I in 3 GC cell lines, AGS, MKN28, and MKN45, by qRT-PCR and western blotting. The results showed that CENP-I was most abundant in MKN28 cells and least abundant in MKN45 cells; therefore, these two cell lines were subjected to CENP-I knockdown and overexpression, respectively, for functional studies (Figure 1E, F). CENP-I knockdown and overexpression were confirmed by western blotting (Figure 1G). Three CENP-I-targeting shRNAs were used for CENP-I knockdown in MKN28 cells, and equal combination of shRNAs with the best knockdown efficacy (shCENP-I-2 and shCENP-I-3) was used for the following studies (Figure 1G).

**Centromere Protein I Knockdown and Overexpression in Gastric Cancer Cell Lines**

CCK-8 assays were used to investigate the role of CENP-I in the proliferation of GC cells. Our results showed that knockdown of CENP-I significantly inhibited the growth of MKN28 cells, while overexpression of CENP-I promoted the proliferation of MKN45 cells (Figure 2A). Using flow cytometry, we confirmed that CENP-I was not involved in the cell cycle regulation of GC cells (data are not shown).

Cell apoptosis was also studied in vitro in GC cell lines using TUNEL and flow cytometry. We found that CENP-I
knockdown or overexpression did not significantly affect apoptosis of MKN28 and MKN45 cells (Figure 2B–D). However, CENP-I knockdown significantly increased the sensitivity of MKN28 cells to CDDP-induced apoptosis (Figure 2B, C) \((P<.001)\).

**Centromere Protein I Promoted Gastric Cancer Cell Migration by Activating Epithelial–Mesenchymal Transition**

Cell migration of GC cells was studied using Transwell assays. The results showed that knockdown of CENP-I significantly inhibited the migration ability of MKN28 cells, while CENP-I overexpression promoted the migration of MKN45 cells (Figure 2E).

EMT is an important biological process involved in the migration and metastasis of cancer cells. We examined the levels of EMT protein markers in GC cells after manipulating CENP-I expression. As anticipated, CENP-I knockdown led to upregulation of the epithelial signature marker E-cadherin, downregulation of the mesenchymal signature markers vimentin and N-cadherin, suggesting inhibition of the EMT pathway, whereas CENP-I overexpression caused downregulation of E-cadherin and upregulation of N-cadherin and the E-cadherin inhibitor ZEB1, suggesting activation of the EMT pathway (Figure 2F). In addition, western blotting of pan-Akt1/2/3 and phosphor-pan-Akt1/2/3 revealed unchanged total AKT levels and reduced phosphorylated AKT in CENP-I-knockdown cells, suggesting inactivation of the AKT pathway (Figure 2F).

**Centromere Protein I Correlation with Gastric Cancer Proliferative Subtype Genetic Signatures**

We analyzed a cohort of 70 primary gastric tumors representing 3 subtypes (invasive, metabolic, and proliferative) from an Australian patient cohort in the GEO database (accession No.: GSE35809). The results showed that CENP-I expression was significantly higher in the proliferative subtype \((n=29)\), which was characterized by high genome instability, TP53 mutation, and low promoter methylation levels, than that in the metabolic \((n=15)\) or invasive subtypes \((n=26)\) (both \(P<.0001)\), whereas there was no difference in CENP-I expression between the metabolic and invasive subtypes \((P=.8649)\) (Figure 2J).

We further analyzed CENP-I promoter methylation in GC patients using the UALCAN database, which showed hypomethylation of the CENP-I promoter in TP53-mutant patients \((P<.05)\) (Figure 2G). Consistently, CENP-I expression in TP53-mutant patients was significantly higher than that in non-mutant patients \((P<.001)\) (Figure 2H). Furthermore, CENP-I promoter methylation levels were significantly lower in male than in female patients \((P<.0001)\) (Figure 2I), which was in accordance with the higher percentage of high-CENP-I cases in male (73.9%) than in female patients (65.2%) in our IHC study (Table 3).

**Discussion**

As faithful chromosome segregation is essential for the maintenance of genomic integrity and stability, the centromere family of proteins has been reported to be involved in many...
pathological events, including cancer. Centromeres are epigenetically defined by CENP-A, a histone H3 variant. Overexpression of CENP-A is common among many cancers and increases the rate of ectopic CENP-A deposition, leading to mitotic defects, centromere dysfunction, and chromosomal instability (CIN). Alterations in CENP-A posttranslational modifications are also linked to chromosome segregation errors and CIN. We reviewed the current literature regarding the reported involvement of CENP proteins in cancer as summarized in Table 5. Consistently, the expression of the CENP family of proteins is upregulated in multiple types of cancers and is associated with advanced cancer features in patients, such as clinical stage, grade, and metastasis. Functional studies have revealed that CENP proteins facilitate cancer cell proliferation, migration, cell cycle transition, EMT, and CIN, and inhibit cell apoptosis. CENP-F and CENP-H are most frequently reported to be associated with cancer. In our study, we found an increased

Figure 2. (A) CCK-8 cell proliferation assay. (B) TUNEL assay of MKN28 cells treated with 20 mg/L CDDP for 24 h. (C) Cell apoptosis measured by TUNEL assay of MKN28 cells. (D) Cell apoptosis measured by flow cytometry of MKN45 cells. (E) Transwell cell migration assay of gastric cancer (GC) cells. (F) Expression of epithelial–mesenchymal transition (EMT) markers and AKT by western blotting in GC cells. (G) Centromere protein I (CENP-I) promoter methylation level and (H) CENP-I expression level in TP53-mutant and nonmutant groups in GC tissues from UALCAN database. (I) CENP-I promoter methylation levels between gender groups in GC tissues from UALCAN database.
transcription of $CENP-A/E/F/H/I/K/P/W$ and $HJURP$ in STAD tissue samples compared to paracancerous normal stomach tissue samples by microarray analysis, and identified increased transcription of the above genes as well as $CENP-B/L/M/N/O/Q/T$ in a dataset of 415 STAD samples sourced from the UALCAN database.

Elevated expression of $CENP-I$ has been described in colorectal cancer (CRC) and estrogen receptor-positive (ER+) breast cancer, but not in ER− breast cancer. It is a strong independent marker for poor patient prognosis and survival of ER+ breast cancer patients and is one of the strongest markers for CIN. Overexpression of $CENP-I$ in breast cancer is not only due to cell cycle-associated expression, but also to chromosome structural gains. In CRC, overexpression of $CENP-I$ is associated with patient tumor clinical staging, tumor depth, lymph node metastasis, distant metastasis, and differentiation, but is not associated with overall patient survival. Downregulation of $CENP-I$ expression suppresses CRC cell migration, invasion, and EMT. In this study, we also confirmed elevated $CENP-I$ mRNA and protein expression in GC tissues. By manipulating $CENP-I$ expression in 2 GC cell lines, we confirmed that $CENP-I$ functions in favoring cell proliferation and cell

### Table 5. Summary of Current Studies of the Involvement of CENP Family of Proteins in Cancer.

| Protein | Cancer type | Clinical relevance | Reference |
|---------|-------------|--------------------|-----------|
| CENP-A  | Epithelial ovarian cancer | Upregulated expression, correlated with pathological grade, stage, poor survival. Independent predictor of OS. | 22 |
|         | Colorectal cancer | Upregulated expression, correlated with aneuploidy. | 23 |
| CENP-E  | Triple-negative breast cancer | Upregulated expression in basal A molecular subtype, correlated with cell viability. | 24 |
| CENP-F  | Pancreatic cancer | Upregulated expression, correlated with poor prognosis of patients. Involved in cell proliferation, migration and EMT, and caused G2/M arrest. Regulated TFN and longevity regulating pathways. | 10 |
| Esophageal squamous cell carcinoma (ESCC) | Upregulated expression, correlated with gender, clinical stage, T classification, and worse OS. Independent prognostic factor for OS. ZOL significantly enhanced sensitivity of ESCC cell lines with high CENP-F expression to cisplatin. | 16 |
| Breast cancer | Upregulated expression, associated with worse OS, reduced MFS, ER negativity, high tumor grade, CIN markers, increased telomerase activity, c-Myc amplification, aneuploidy, VEGFR2, phosphorylated Ets-2, and Ki67. Independent predictor of worse breast cancer-specific survival and OS. | 17 |
| Head and neck squamous cell carcinoma | Upregulated expression. | 18 |
| CENP-H  | Uterine cervical cancer | Upregulated expression, associated with stage and poor survival. Independent prognostic markers for patient survival. | 19 |
| Gastric cancer | Upregulated expression, correlated with tumor size, depth of infiltration, lymph node metastasis, distant metastasis, and UICC staging. CENP-H and Ki67 expression was a more valuable independent prognostic predictor for patients’ survival. Involved in GC cell growth, proliferation, and clonogenic ability. | 11 |
| Hypopharyngeal squamous cell carcinoma | Upregulated expression, associated with advanced cancer stage and alcohol history, and short RFS. Independent predictor of RFS. Promoted HSCC cell proliferation and inhibited apoptosis. | 20 |
| Breast cancer | Upregulated expression, associated with clinical stage, T classification, N classification, Ki-67, and shorter OS. Independent prognostic indicator for patient survival. | 21 |
| Tongue cancer | Upregulated expression, associated with clinical stage, T classification. Promoted cancer cell proliferation and downregulated the expression of Survivin. | 12 |
| CENP-I  | ER+ breast cancer | Upregulated expression. Independent marker for poor patient prognosis and survival. Regulated by E2F. One of the strongest markers for CIN. | 25 |
| Colorectal cancer | Upregulated expression, associated with clinical stage, tumor depth, lymph node metastasis, distant metastasis, and differentiation. Promoted tumor cell migration, invasion, and EMT. | 13 |
| CENP-K  | Ovarian cancer | Upregulated expression, associated with poor prognoses. Improved the sensitivity of CA125 or HE4 for predicting clinical outcomes of ovarian cancer patients. | 26 |
| CENP-N  | Oral squamous cell carcinoma | Upregulated expression, correlated with tumor growth. Promoted cell proliferation by facilitating G1/S transit, downregulating p21 (Cip1) and p27(Kip1), and upregulating cyclin D1, CDK2, and CDK4. Calcitriol controlled CENP-N expression, leading to inhibition of tumor growth. | 14 |
| CENP-W  | Hepatocellular carcinoma | Upregulated expression, associated with worse prognosis. Promoted cell proliferation, migration, invasion, and G2/M transit, and inhibited apoptosis. | 15 |

Abbreviations: CIN, chromosomal instability; EMT, epithelial–mesenchymal transition; MFS, metastasis-free survival; OS, overall survival; RFS, relapse-free survival.
migration and inhibiting cell apoptosis. CENP-I also promoted EMT, possibly by activating the AKT/mTOR pathway, which was in accordance with its reported correlation with lymph node metastasis.

In this study, we found that CENP-I and HER2 were both independent prognostic factors of GC, and that elevated CENP-I expression was associated with advanced tumor stage, lymph node invasion, HER2 amplification, and intestinal Lauren subtype. These findings were in accordance with previous reports. Li et al.27 recently found that HER2 overexpression is an independent predictor of prognosis in GC patients, and that HER2 amplification and Lauren intestinal type are associated with poor prognosis in GC patients. Another study found that HER2 overexpression is positively correlated with the proportion of the intestinal Lauren subtype.28 Together, our results confirmed the association between elevated CENP-I expression and malignant characteristics and poorer prognosis of GC patients, and the correlation between CENP-I expression and HER2/intestinal Lauren subtype.

A study by Lei et al. identified 3 subtypes of STAD based on the gene expression patterns of 248 gastric tumors: proliferative, metabolic, and mesenchymal.29 Tumors of the proliferative subtype had upregulated gene expression associated with mitotic cell cycle and DNA replication, low tumor grade, intestinal Lauren classification (73.6%), high levels of copy number alteration, gene amplification of CCNE1, MYC, ERBB2, and KRAS; high-frequency TP53 mutations and DNA hypomethylation; and activation of the E2F, MYC, and RAS pathways.29 Our study identified significantly higher CENP-I expression in the proliferative subtype than in the other 2 subtypes from an Australian patient cohort in the GEO database (accession No.: GSE35809). CENP-I has also been reported to be an E2F target gene and a strong marker for CIN.12 In addition, our IHC and bioinformatic studies confirmed a correlation between high CENP-I expression and intestinal Lauren classification, HER2 (encoded by ERBB2) amplification, and TP53 mutation in GC samples. Therefore, CENP-I hyperexpression is closely associated with the genetic signatures of the proliferative GC subtype and is one of the contributing factors to the development and malignancy of GC.

In conclusion, this study confirmed the upregulated expression of the CENP family of proteins, especially CENP-I, in GC. CENP-I was found to be an independent prognostic marker that is unfavorable for patient survival. CENP-I promotes GC cell proliferation, inhibits cell apoptosis, facilitates cell migration, and activates the EMT pathway. It was correlated with the genetic signatures of the proliferative subtype of GC and could be a potential target for anticancer therapy in GC patients.

**Ethics Statement**

Ethical approval to report this case series was obtained from the Ethics Committee of Yantai Yuhuangding Hospital, China (IRB No.: YHD 2018-150). Written informed consent was obtained from the patients for their anonymized information to be published in this article.

**Declaration of Conflicting Interests**

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**Data Availability**

Data analyzed and generated in this study are not publicly available because of privacy restrictions; however, they can be provided on reasonable request to the corresponding author.

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**Supplemental Material**

Supplemental material for this article is available online.

**References**

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jamal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424. Doi: 10.3322/caac.21492
2. Hamashima C. The burden of gastric cancer. Ann Transl Med. 2020;8(12):734. Doi: 10.21037/atm.2020.03.166
3. Avital I, Pisters PWT, Kelsen DP, Willett CG. (2011). Cancer of the stomach. In Devita V. T., Lawrence T. S., Rosenberg S. A. (Eds.), DeVita, Hellman, and Rosenberg's Cancer: Principles and Practice of Oncology (9th edn., pp. 924-954). Lippincott Williams & Wilkins.
4. Schwartzman JM, Sotillo R, Benezra R. Mitotic chromosomal instability and cancer: mouse modelling of the human disease. Nat Rev Cancer. 2010;10(2):102-115. Doi: 10.1038/nrc2781
5. Perpelescu M, Fukagawa T. The ABCs of CENPs. Chromosoma. 2011;120(5):425-446. Doi: 10.1007/s00412-011-0330-0
6. Lin C, Wang J, Wang Y, et al. GRP78 Participates in PCA3-regulated prostate cancer progression. Anticancer Res. 2017;37(8):4303-4310. Doi: 10.21873/anticancer.11823
7. Yang Y, Lu T, Li Z, Lu S. FGFR1 Regulates proliferation and metastasis by targeting CCND1 in FGFR1 amplified lung cancer. Cell Adh Migr. 2020; 14(1): 82-95. Doi: 10.1080/19336918.2020.1766308
8. Chandra Shekar DS, Bashel B, Balasubramanayam SAH, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia. 2017;19(8):649-658. Doi: 10.1016/j.neo.2017.05.002
9. Mahlke MA, Nechemia-Arbel Y. Guarding the genome: cENP-A-chromatin in health and cancer. Genes. 2020;11(7):810. Doi: 10.3390 gen11070810
10. Chen H, Wang X, Wu F, et al. Centromere protein F is identified as a novel therapeutic target by genomics profile and contributing to the progression of pancreatic cancer. *Genomics*. 2021;113(1 Pt 2):1087-1095. Doi: 10.1016/j.ygeno.2020.10.039

11. He WL, Li YH, Yang DJ, et al. Combined evaluation of centromere protein H and Ki-67 as prognostic biomarker for patients with gastric carcinoma. *Eur J Surg Oncol*. 2013;39(2):141-149. Doi: 10.1016/j.ejso.2012.08.023

12. Liao WT, Yu CP, Wu DH, et al. Upregulation of CENP-H in tongue cancer correlates with poor prognosis and progression. *J Exp Clin Cancer Res*. 2009;28(1):74. Doi: 10.1186/1756-9966-28-74

13. Ding N, Li R, Shi W, He C. CENPI is overexpressed in colorectal cancer and regulates cell migration and invasion. *Gene*. 2018;674:80-86. Doi: 10.1016/j.gene.2018.06.067

14. Oka N, Kasamatsu A, Endo-Sakamoto Y, et al. Centromere protein N participates in cellular proliferation of human oral cancer by cell-cycle enhancement. *J Cancer*. 2019;10(16):3728-3734. Doi: 10.7150/jca.32281

15. Zhou Z, Zhou Z, Huang Z, et al. Histone-fold centromere protein W (CENP-W) is associated with the biological behavior of head-and-neck carcinoma cells. *Bioengineered*. 2020;11(1):729-742. Doi: 10.1080/21655979.2020.1787776

16. Mi YJ, Gao J, Xie JD, et al. Prognostic relevance and therapeutic implications of centromere protein F expression in patients with esophageal squamous cell carcinoma. *Dis Esophagus*. 2013;26(6):636-643. Doi: 10.1111/dote.12002

17. O’Brien SL, Fagan A, Fox EJ, et al. CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. *Int J Cancer*. 2007;120(7):1434-1443. Doi: 10.1002/ijc.22413

18. de la Guardia C, Casiano CA, Trinidad-Pinedo J, Baez A. CENP-F gene amplification and overexpression in head and neck squamous cell carcinomas. *Head Neck*. 2001;23(2):104-112. Doi: 10.1002/1097-0347(200102)23:2<104::aid-hed1005>3.0.co;2-0

19. Weng MY, Li L, Hong SJ, Feng SY. Clinical significance of CENP-H expression in uterine cervical cancer. *Cancer Biol Med*. 2012;9(3):192-196. Doi: 10.7497/j.issn.2095-3941.2012.03.007

20. Wang JX, Zhang YY, Yu XM, et al. Role of centromere protein H and Ki67 in relapse-free survival of patients after primary surgery for hypopharyngeal cancer. *Asian Pac J Cancer Prev*. 2012;13(3):821-825. Doi: 10.7314/apjcp.2012.13.3.821

21. Liao WT, Feng Y, Li ML, et al. Overexpression of centromere protein H is significantly associated with breast cancer progression and overall patient survival. *Chin J Cancer*. 2011;30(9):627-637. Doi: 10.5732/cjc.010.10599

22. Qiu JJ, Guo JJ, Lv TJ, et al. Prognostic value of centromere protein-A expression in patients with epithelial ovarian cancer. *Tumour Biol*. 2013;34(5):2971-2975. Doi: 10.1007/s13277-013-0860-6

23. Tomonaga T, Matsushita K, Yamaguchi S, et al. Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res*. 2003;63(13):3511-3516.

24. Kung PP, Martinez R, Zhu Z, et al. Chemogenetic evaluation of the mitotic kinsein CENP-E reveals a critical role in triple-negative breast cancer. *Mol Cancer Ther*. 2014;13(8):2104-2115. Doi: 10.1158/1535-7163.MCT-14-0083-T

25. Thangavelu PU, Lin CY, Vaidyanathan S, et al. Overexpression of the E2F target gene CENPI promotes chromosome instability and predicts poor prognosis in estrogen receptor-positive breast cancer. *Oncotarget*. 2017;8(37):62167-62182. Doi: 10.18632/oncotarget.19131

26. Lee YC, Huang CC, Lin DY, et al. Overexpression of centromere protein K (CENPK) in ovarian cancer is correlated with poor patient survival and associated with predictive and prognostic relevance. *PeerJ*. 2015;3:e1386. Doi: 10.7717/peerj.1386

27. Li F, Meng G, Tan B, et al. Relationship between HER2 expression and tumor interstitial angiogenesis in primary gastric cancer and its effect on prognosis. *Pathol Res Pract*. 2020;215:153280. Doi: 10.1016/j.prrp.2020.153280

28. Xi Y, Xu C, Liu Y, et al. The age variation of HER2 immunohistochemistry positive rate in biopsy specimens of gastric cancer. *Pathol Res Pract*. 2020;216(4):152882. Doi: 10.1016/j.prrp.2020.152882

29. Lei Z, Tan IB, Das K, et al. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. *Gastroenterology*. 2013;145(3):554-565. Doi: 10.1053/j.gastro.2013.05.010