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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/I/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; RIPA, 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

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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, 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-H/I/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: gcTCTTCTTTACATCAACCAT, shCENP-I-2: AGGCTTTGTTGTCACTGTATA, shCENP-I-3: TTGCAA ATGGCAGTGGGATAT) 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 $2^{-\hat{\Delta}\Delta Ct}$ method. All experiments were performed in triplicate. The primer sequences were as follows: CENP-I-F, 5′-CAGATGGGCTCAGT GCTAAA-3′, CENP-I-R: 5′-ATGGTATCCAGGAAGTTGG TAAA-3′, β-Tubulin-F: 5′-GGACCGATCTCTGTGTACTA-3′, β-Tubulin-R: 5′-CTTTGGCCCAGTTGTTACCT-3′.

Western Blotting

Total protein was extracted from cells lysed with radio-immunoprecipitaion 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	TAGTTTGTGAGTTACTCATGTGACTATTTGAGGATTTTGAAAACATCAGATTTGCTGTGG	NM_001809	3.5259418	1.5203211
CENP-E	A_23_P253524	CTGAGTGCAAAACTCAGTAGACTCCTCTTTGTCACTTCTCTGGAGATCCAGCATTCCTTA	NM_001813	2.9304266	1.1442649
CENP-F	A_24_P96780	GCTGGAGATAGACCTTTTAAAGTCTAGTAAAGAAGAGCTCAATAATTCATTGAAAGCTAC	NM_016343	3.9159503	1.1696385
CENP-H	A_23_P110802	TTTACATTTCTGGCAATCTCAACTCTTATTTGGAATACTTCTGTGCATTTGTCTGTCCAC	NM_022909	2.822752	1.091639
CENP-I	A_33_P3221313	TCCCAGAGCAGAGGGCATAAACTGCAACAATCAATATTAAATGAATGTTGACATAAACTG	NM_006733	5.23768	−1.0817137
CENP-K	A_23_P155989	TTGAGGTAATATCTGAGGTGGCATAATTTAAAAATATTTAGCAAATTTGTTTCATATATA	NM_022145	2.5990665	−1.0310805
CENP-P	A_33_P3245321	TGGAATCGAAGCTGCTCTGGAAAGCCTGATAAAATCGCTTTGTGCAGAGGAGAACAACTA	NM_001012267	2.2836795	1.7405572
CENP-W	A_24_P462899	GAAGAGCAGAGGTTAGAAGTCAAAGAACATATTCTTGAAAGTTATGATGCATTCTTTTGG	NM_001012507	2.4428961	1.603932
HJURP	A_33_P3807062	CATCAGAGATAACCTCGAGTTCTTGGTGTAGAAATTATGTGAATAAAGTTGCTCAATTAG	NM_018410	4.137667	−1.3971884

Abbreviations: STAD, stomach adenocarcinoma; SRCC, signet ring cell carcinoma.

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		
	STAD	Normal	STAD versus normal	*P* value of expression	*P* value of effect on survival
CENP-A	11.347 (7.068, 16.245)	2.783 (0.275, 3.947)	4.08	1.62E-12	.27
CENP-B	72.774 (53.084, 97.73)	66.13 (2.188, 77.384)	1.10	8.04E-04	.83
CENP-E	9.498 (5.534, 14.774)	0.821 (0.505, 1.025)	11.57	1.62E-12	.47
CENP-F	14.511 (7.933, 23.092)	1.418 (0.378, 2.043)	10.23	<1E-12	.80
CENP-H	9.399 (6.161, 13.107)	3.299 (2.481, 4.298)	2.85	<1E-12	.78
CENP-I	2.722 (1.496, 4.508)	0.414 (0.202, 0.548)	6.57	<1E-12	.19
CENP-K	8.139 (5.354, 11.034)	1.87 (0.437, 2.887)	4.35	<1E-12	.65
CENP-L	8.215 (5.88, 10.786)	2.391 (2.088, 2.877)	3.44	1.62E-12	.083
CENP-M	21.483 (13.304, 31.521)	10.462 (1.023, 15.016)	2.05	9.29E-12	.052
CENP-N	14.985 (9.885, 19.7)	5.299 (4.142, 6.411)	2.83	<1E-12	.48
CENP-O	9.959 (6.384, 3.66)	2.981 (1.043, 4.053)	3.34	1.62E-12	.83
CENP-P	3.289 (2.455, 4.354)	1.41 (1.056, 1.906)	2.33	<1E-12	.45
CENP-Q	5.079 (3.687, 7.101)	2.297 (1.444, 2.993)	2.21	<1E-12	.57
CENP-T	14.816 (12.135, 17.988)	13.184 (8.701, 15.39)	1.12	7.86E-03	.77
CENP-W	38.184 (25.445, 55.522)	11.568 (6.849, 17.064)	3.3	1.62E-12	.96
HJURP	10.719 (6.839, 16.246)	1.853 (0.245, 2.602)	5.78	1.62E-12	.14

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

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[6] 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 $\times 200$ 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 ($\times 400$) 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 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% CO_2 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 (2×10^3-1×10^4 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[7] with 4×10^4 cells seeded into the upper chamber.

TUNEL

To assess cell apoptosis, 1×10^5 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 (Thermofisher 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

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 Cycletest™ 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

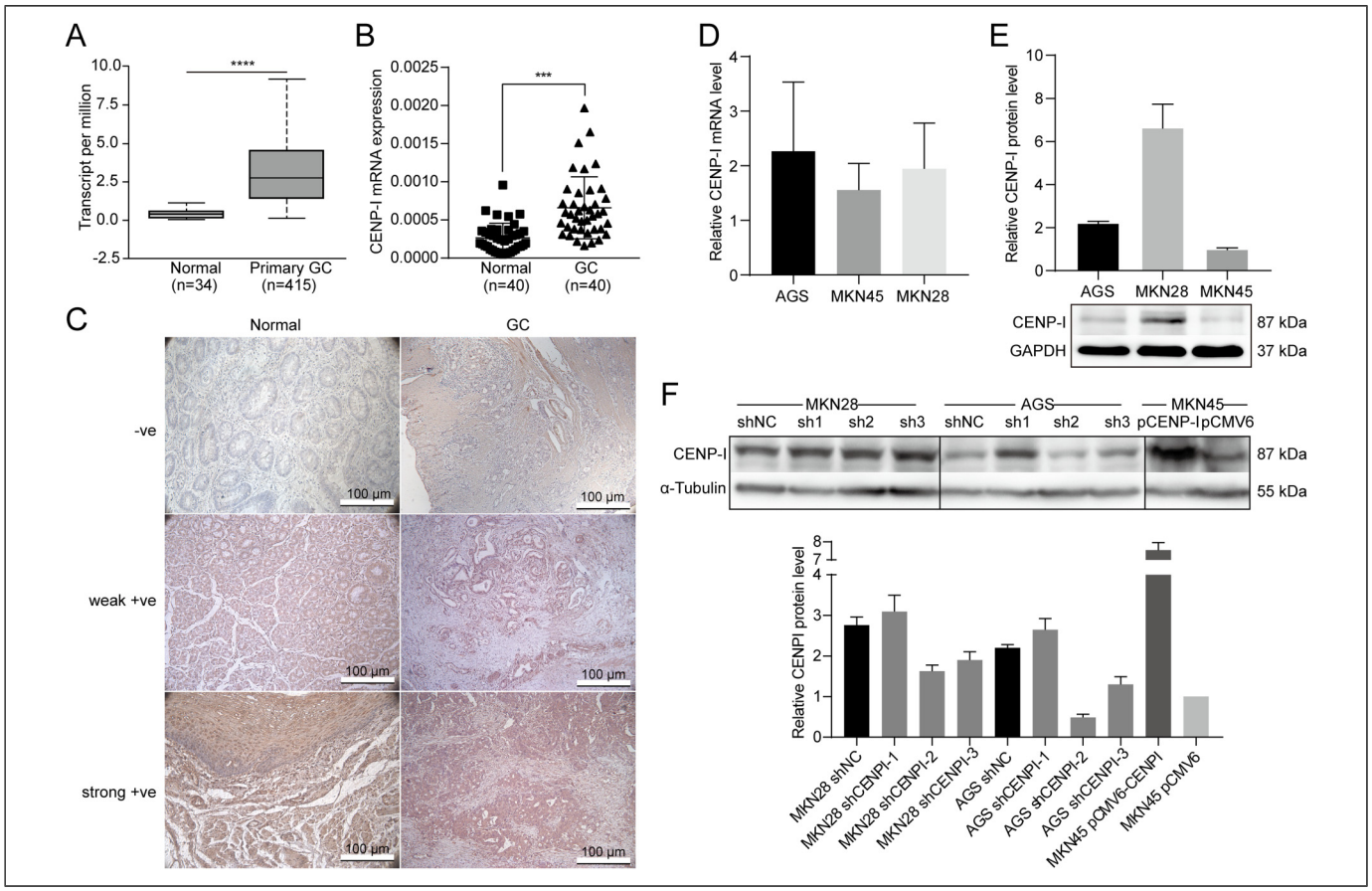

Figure 1. (A) Centromere protein I (*CENP-I*) mRNA expression elevated in gastric cancer (GC) tissues compared to normal gastric tissues ($P<0.0001$) from UALCAN database. (B) real-time quantitative polymerase chain reaction (RT-qPCR) revealed significantly higher *CENP-I* mRNA expression in 40 GC tissues than that in 40 paired normal gastric tissues ($P<.0001$). (C) Representative images of immunohistochemical staining for CENP-I in GC and normal gastric tissues. (D) *CENP-I* mRNA abundance in 3 GC cell lines measured by quantitative real-time PCR (qRT-PCR). (E) CENP-I protein expression in 3 GC cell lines measured by western blotting. (F) Western blotting showed knockdown and overexpression of CENP-I in 3 GC cell lines.

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

Characteristics	N	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				.575
Female	23	15 (21.7)	8 (11.6)	
Male	46	34 (49.3)	12 (17.4)	
Clinical stage				.0319*
I to II	30	17 (24.6)	13 (18.9)	
III to -IV	39	32 (46.4)	7 (10.1)	
Tumor grade				.170
G1 to G2	44	34 (49.3)	10 (14.5)	
G3 to G4	25	15 (21.7)	10 (14.5)	
Tumor depth				.547
T1 to T2	16	11 (15.9)	6 (8.7)	
T3 to T4	53	38 (55.1)	14 (20.3)	
Lymph node invasion				.0299*
Yes	45	36 (52.2)	9 (13.0)	
No	24	13 (18.8)	11 (16.0)	
Tumor size (cm)				.430
1 to 4.5	32	21 (30.4)	11 (16.0)	
>4.5	37	28 (40.6)	9 (13.0)	
HER2				.0392*
−	49	31 (44.9)	18 (26.1)	
+	20	18 (26.1)	2 (2.9)	
Lauren subtype				.0069**
Intestinal	43	35 (50.7)	7 (10.1)	
Diffuse	26	14 (20.3)	13 (18.9)	

Abbreviation: CENP-I, cemtromere protein I. *: $P<0.05$, **: $P<0.01$.

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 centromeric 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 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 ($r^2 = .319$, $P = .008$), HER2 overexpression ($r^2 = .449$, $P<.001$), and intestinal Lauren subtype ($r^2 = .339$, $P = .004$). 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.

Centromere Protein I Knockdown and Overexpression in Gastric Cancer Cell Lines

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 Promoted Gastric Cancer Cell Proliferation and Cisplatin-Induced Apoptosis in Vitro

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

Table 4. Cox regression Analysis of Associations of Centromere Protein I Expression with Survival and Clinicopathological Characteristics.

		Univariate analysis			Multivariate analysis[a]		
		Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
Age	Continuous	1.02	0.99-1.05	.284			
Gender	Male/female	1.78	0.90-3.55	.100			
Tumor stage	I	1.00		**.005**	-	-	.245
	II	2.44	0.53-11.14	.250	-	-	.424
	III	5.81	1.37-24.68	**.017**	-	-	.678
	IV	9.30	1.86-46.52	**.007**	-	-	.113
Tumor grade	G1	1.00		.736			
	G2	1.13	0.27-4.77	.871			
	G3	1.42	0.37-6.15	.640			
Tumor depth	T1	1.00		.517			
	T2	0.75	0.17-3.37	.711			
	T3	1.51	0.43-5.31	.519			
	T4	1.58	0.47-5.27	.457			
Lymph node invasion	Yes/No	2.17	1.09-4.31	**.028**	-	-	.416
Tumor size	≥4.5/<4.5	2.97	1.54-5.75	**.001**	2.49	1.26-4.79	**.008**
Tumor size	Continuous	1.13	1.04-1.24	**.007**	-	-	.430
HER2	+/−	0.31	0.16-0.57	**.000**	0.46	0.24-0.92	**.027**
Lauren subtype	Diffused/intestinal	0.49	0.25-0.95	**.036**	-	-	.242
CENP-I	High/low	3.83	1.61-9.12	**.002**	2.66	1.00-6.24	**.050**

Abbreviation: CENP-I, cemtromere protein I. [a]Selection methods: stepwise forward regression. Age, gender, tumor stage, grade, and depth were excluded from multivariate analysis because of no significant effect in the univariate analysis.

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

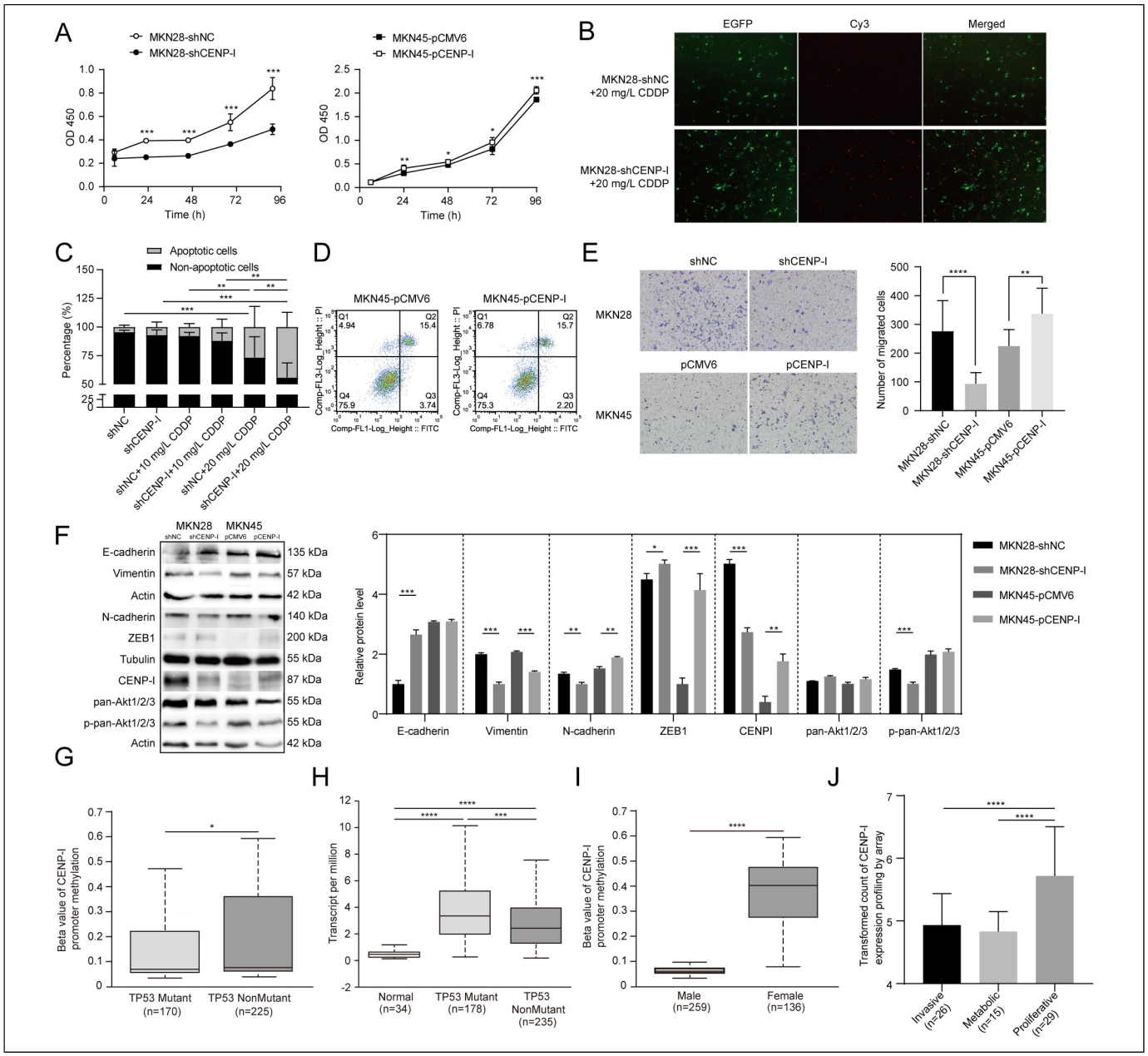

Figure 2. (A) CCK-8 cell proliferation assay. (B) TUNEL assay of MKN28 cells treated with 20 mg/L CDDP for 24 h. (C) Cell apoptosis measured by TUNEL assay of MKN28 cells. (D) Cell apoptosis measured by flow cytometry of MKN45 cells. (E) Transwell cell migration assay of gastric cancer (GC) cells. (F) 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.

pathological events, including cancer. Centromeres are epigenetically defined by CENP-A, a histone H3 variant.[9] 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.[9] 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.[10-15] CENP-F and CENP-H are most frequently reported to be associated with cancer.[10-12,16-21] In our study, we found an increased

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 TNF 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.

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.[25] Overexpression of CENP-I in breast cancer is not only due to cell cycle-associated expression, but also to chromosome structural gains.[25] 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.[13] Downregulation of CENP-I expression suppresses CRC cell migration, invasion, and EMT.[13] 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

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.

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