High expression of centromere protein N as novel biomarkers for gastric adenocarcinoma

Xiao Wang[1], Ke Zhang[1], Cun Fu[1], Fei Wu[1], Jun Zhang[1], Bin Han[2], Hai Pan[1], and Lan Luan[1]

[1]Central Hospital affiliated to Shenyang Medical College
[2]Shengjing Hospital of China Medical University

October 26, 2022

Abstract

Background: The role and mechanism of centromeric protein N (CENPN), which has been associated with the development of various cancer types, are yet unclear in Gastric adenocarcinoma (STAD). Methods: Data from The Cancer Genome Atlas and Genotype Tissue Expression were used to examine the expression of CENPN in STAD and neighboring tissues. Xiantao Academic was used to perform Gene Ontology(GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) enrichment analysis on CENPN. By reviewing TCGA database, the relationship between CENPN expression and immune cell infiltration was assessed. The expression of CENPN in STAD and surrounding tissues was confirmed by immunohistochemical staining, and the correlation between CENPN expression and clinicopathological characteristics was examined. CENPN was depleted in AGS cells with siRNAs, and its impact on proliferation was measured by CCK-8 and EdU assays. Following siRNA transfection, flow cytometry was performed to identify cell cycle and apoptotic alterations in AGS cells. Results: CENPN was highly expressed in STAD tissues. The degree of invasion, TNM stage, and lymph node metastases were all substantially linked with CENPN expression. GO|KEGG Enrichment analysis revealed that CENPN was essential for the cell cycle, DNA replication, chromosomal segregation, and nuclear division, among other important signaling pathways. Further investigation revealed a positive correlation between CENPN expression and Th2 cells and NK CD56dim cells and a negative correlation between CENPN expression and mast cells , pDC cells,NK cells and B cells. When CENPN expression in AGS cells was knocked down, cell proliferation dramatically reduced, and the percentage of cells in the S and G2-M phases decreased significantly. In addition, compared to the control group, the proportion of apoptotic AGS cells significantly increased after downregulating the expression level of CENPN. Conclusion: According to our data, CENPN acts as an oncogene in STAD and may be a viable therapeutic target.

High expression of centromere protein N as

novel biomarkers for gastric adenocarcinoma

Xiaojie Wang[1,2], Keyuan Zhang[1,2], Cun Fu [1,2], Fei Wu[1], Junjie Zhang[1], Bin Han[3], Hai Pan[4], Lan Luan[1*]

[1] Department of Pathology, Central Hospital Affiliated to Shenyang Medical College, Shenyang, China.

[2] Basic Medical School, Shenyang Medical College, Shenyang, China.

[3] Department of Urology, Shengjing Hospital of China Medical University, Shenyang, China.

[4] Central Laboratory, Central Hospital Affiliated to Shenyang Medical College, Shenyang, China.

*** Correspondence:** Lan Luan luanlan33@symc.edu.cn

Keywords: CENPN; gastric adenocarcinoma; proliferation; cycle; apoptosis.

Abstract

Background: The role and mechanism of centromeric protein N (CENPN), which has been associated with the development of various cancer types, are yet unclear in Gastric adenocarcinoma (STAD).

Methods: Data from The Cancer Genome Atlas and Genotype Tissue Expression were used to examine the expression of CENPN in STAD and neighboring tissues. Xiantao Academic was used to perform Gene Ontology(GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) enrichment analysis on CENPN. By reviewing TCGA database, the relationship between CENPN expression and immune cell infiltration was assessed. The expression of CENPN in STAD and surrounding tissues was confirmed by immunohistochemical staining, and the correlation between CENPN expression and clinicopathological characteristics was examined. CENPN was depleted in AGS cells with siRNAs, and its impact on proliferation was measured by CCK-8 and EdU assays. Following siRNA transfection, flow cytometry was performed to identify cell cycle and apoptotic alterations in AGS cells.

Results: CENPN was highly expressed in STAD tissues. The degree of invasion, TNM stage, and lymph node metastases were all substantially linked with CENPN expression. GO|KEGG Enrichment analysis revealed that CENPN was essential for the cell cycle, DNA replication, chromosomal segregation, and nuclear division, among other important signaling pathways. Further investigation revealed a positive correlation between CENPN expression and Th2 cells and NK CD56dim cells and a negative correlation between CENPN expression and mast cells , pDC cells,NK cells and B cells. When CENPN expression in AGS cells was knocked down, cell proliferation dramatically reduced, and the percentage of cells in the S and G2-M phases decreased significantly. In addition, compared to the control group, the proportion of apoptotic AGS cells significantly increased after downregulating the expression level of CENPN.

Conclusion: According to our data, CENPN acts as an oncogene in STAD and may be a viable therapeutic target.

Introduction

Gastric cancer is a global health problem with a high fatality rate because it is frequently discovered at an advanced stage. It is also the third most common cause of cancer-related death[1]. Therefore, early identification and diagnosis are critical for the treatment of stomach cancer. Currently, the methods often adopted for the timely identification of stomach cancer have several flaws. For example, tumor marker specificity and sensitivity are insufficient[2, 3], gastroscopy screening is difficult to implement, and imaging examination plays a limited role in the identification of early gastric cancer[4]. Therefore, the discovery of novel markers with high specificity and sensitivity is critical.

The centromere is a combination of DNA and proteins. Although DNA sequences differ greatly among species, protein components are relatively conserved[5, 6]. These highly conserved proteins are called centromere-associated proteins (CENPs)[7]. According to previous studies, CENPs affect the normal separation of sister chromatids and maintain chromosome stability, making them an important factor in the occurrence and progression of tumors[7-10]. The constitutive centromere-associated network (CCAN) comprises 16 centromere proteins that can be divided into five functional complexes: CENPC, CENPL/N, CENPH/I/K/M, CENPT/W/S/X, and CENPO/P/Q/R/U[11, 12]. CCAN is essential for the separation of chromatids[13, 14]. CENPN interacts with CENPL to form a CENPL/N complex, which is essential for CCAN protein assembly. Not only does it recognize CENPA nucleosomes, but also recruits CENPH/I/K/M complexes. Furthermore, CENPL/N can interact with CENPC, which is required for stabilization of CENPC on kinetosomes[15, 16].

CENPN is a member of the centromeric protein family that has been linked to the occurrence and progression of glioma, liver cancer, and lung cancer[17-19]. We used bioinformatics, immunohistochemistry, and functional

studies To investigate the expression of CENPN in STAD and its effect on the proliferation, cell cycle, and apoptosis of AGS cells.

Materials and methods

Source of the specimen

The study included 76 wax block specimens surgically resected from confirmed patients with STAD at the Department of Pathology, Affiliated Central Hospital of Shenyang Medical College, between June 2014 and July 2016. Paracellular tissue samples from the same patients, including 53 males and 23 females,were chosen as the control group. The ages of the patients ranged from 36 to 95 years, with a mean age of 66 years. None of the patients receive radiotherapy or chemotherapy before surgery. This study was approved by the Medical Ethics Committee of the Shenyang Medical College, China.

Data gathering and analysis

Differences in CENPN expression between STAD and normal tissues were evaluated using TCGA (https://cancergenome.nih.gov) and GTEX databases.The TCGA database was used to obtain RNA sequencing data and clinical follow-up information for patients with STAD.

CENPN functional enrichment analysis and screening of co-expressed genes in STAD

TCGA-STAD data were used to perform a Pearson correlation study in CENPN and other mRNAs in gastric cancer. For enrichment analysis to assess the function of CENPN, the 300 genes with the highest positive correlations with CENPN were chosen. GO and KEGG pathway analyses were performed using Xiantao Academic.

Immune infiltration analysis

The Xiantao Academic and single-sample gene set enrichment analysis were used to examine the link between CENPN expression in STAD and 24 different types of immune cells (ssGSEA). Additionally, the relationship between CENPN expression and Th2, mast cells, Mast cells , pDC ,NK cells ,and B cells was examined using Spearman's correlation.

Immunohistochemical staining

After roasting, dewaxing, antigen repair, and endogenous peroxidase blocker, paraffin sections were incubated with rabbit polyclonal anti-CENPN antibody (1:400 dilution,Proteintech,IL,USA) and kept in a refrigerator at 4 °C overnight. The next day, the sections were left at 25°C for 30 min, followed by incubation with the secondary antibody (Fuzhou Maixin Biotechnology Development Co, Ltd,China) at 25°C. Sections were then stained with DAB and hematoxylin, dehydrated with absolute alcohol for 10 min, and sealed with neutral gum.

Evaluating outcomes: The cytoplasm and nucleus were the main sites for CENPN expression. Two pathologists performed a blind review of the immunohistochemical results. Five visual fields at random were chosen, and the staining intensity and percentage were assessed and assigned a semi-quantitative score. The percentage scores given for stained cells were 0 (4% or less), 1 (5%–24%), 2 (25%–49%), 3 (50%–74%), or 4 (75%).The staining intensities were assigned 0 (no staining), 1 (light yellow particles), 2 (yellow), and 3. (dark-yellow particles). The staining intensity score was multiplied by the percentage score of positively

stained cells in each tumor sample to yield a total score ranging from 0 to 12. A score of less than 3 was considered negative, whereas a score of 3 or higher wasconsidered positive.

Cell culture and transfection

AGS cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were routinely sub-cultured in F12 medium (Gibco, USA) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in a cell incubator with 5% CO2.Transfection of siRNAs was carried out in accordance with the manufacturer's instructions using the Lipo3000 reagent (Life Technology Company). After 48 h of transfection, the depletion of CENPN protein was examined by western blotting in gastric cancer cells that were in the logarithmic growth phase.

Western blot analysis

Cells were lysed using RIPA buffer according to the manufacturer's protocol. Protein (30μg/lane) was added to the 10% SDS-PAGE gel and then transferred to a membrane, then transferred to a PVDF membrane, blocked with 5% skim milk for 1 hour in TBST, They are incubated with primary antibody (Proteintech,IL,USA) at 4°C overnight. Membranes were then washed three times andincubated with secondary antibody (Fuzhou Maixin Biotechnology Development Co, Ltd,China) for 1 h. The signal was detected using an enhanced chemiluminescence Western blot assay kit.

CCK-8 experiments

In a 96-well plate, 5000 cells were seeded into each well. To each well, 10 μl of CCK-8 (APExBio, USA) were added at 0, 24, 48, 72, and 96 h. A microplate reader (TECAN Infinite M200pro) was used to measure the optical density at 450 nm following a 1h incubation at 37 °C, and a cell growth curve was drawn.

EdU cell proliferation assay

Cells were seeded in 24-well plates at the logarithmic growth stage and cultured overnight in incubators. Transfection was performed accordantly. Each well was labelled with diluted EdU (Guangzhou Ruibo Biotechnology Co.,Ltd., China) (1:5000 dilution) and incubated for 48 h at 37 °C in a 5% CO2 incubator. The cells were fixed, penetrant added, and stained. Hoechst 33342 was then added to each well at a dilution of 1:1000. After 15 min of shaking at room temperature in the dark, the dye solution was removed, and the cells were washed with phosphate-buffered saline. Finally, an inverted Microscope was used for observation, and five different photographic fields were picked At random.

Cell cycle detection

Cell cycle progression was detected using a cell cycle detection kit (BD Pharmingen, USA) according to the manufacturer's instructions. Cells in 6-well plates were transfected for 48 h and fixed in 75% ethanol, labeled with propidium iodide, and analyzed by flow cytometry (BD FACSCalibur). Each group consisted of three replicates.

Detection of cell apoptosis

Apoptosis was measured using an apoptosis detection kit (Vazyme). Cells were transfected for 48 h in 6-well plates, stained with annexin V and propidium iodide, and analyzed using flow cytometry (BD FACSCalibur).

Statistical analysis

GraphPad Prism 8 and SPSS 20.0 were both used for statistical analysis in this study. Every experiment included at least three independent replicates. Student's t-test was used to calculate statistical significance and results were reported as mean ± standard deviation. Chi-square analysis was used to examine count data. $P < 0.05$ was considered statistically significant.

Results

Expression of CENPN in STAD tissue

Using the GEPIA database, we first examined the expression of CENPN in STAD in TCGA and GTEx databases. STAD tissues had considerably higher levels of CENPN mRNA expression than their neighboring tissues (Figure 1A, $P < 0.05$). We used the TCGA database to assess the expression of CENPN in STAD paired samples, and the results revealed that in 27 samples, the expression of CENPN in STAD was higher than that in matched normal tissues (Figure 1B, $P < 0.001$). In addition, the ROC curve showed that CENPN expression had good predictive ability, with an area of 0.950 under the curve (95% confidence interval [CI]=0.926–0.973), and STAD tissues could be distinguished from normal tissues (Figure 1C).

To further validate CENPN expression in STAD, immunohistochemical stainingwas performed on 76 tumor specimens and adjacent paracellular tissues to assess the differential expression of CENPN. CENPN was highly expressed in STAD tissues, with low or no expression in paracellular tissues, and its positive expression sites were primarily in the cytoplasm and nucleus (Figure 1D). The positivity rate in STAD tissues was 76.3%, whereas it was 18.4% in adjacent tissues (Table 1).

Relationship between CENPN expression in STAD and clinicopathological parameters

We investigated the relationship between CENPN expression and various clinicopathological parameters in patients with STAD to better understand the significance and possible molecular mechanisms of CENPN expression in the development of STAD. The expression of CENPN was significantly different in STAD patients with varying degrees of invasion, TNM stage, and lymph node metastasis ($P < 0.05$). However, there was no statistically significant difference in CENPN expression according to sex, age, tumor size, or degree of differentiation (Table 2) .

CENPN functional enrichment analysis and co-expression gene screening in STAD

To further investigate the functions and pathways affected by CENPN, we used TCGA data to examine the correlation between CENPN and other genes in STAD. The top 300 genes most strongly associated with CENPN were chosen for enrichment analysis, and the top 20 genes are displayed in the heat map (Figure 2A). Using the Xiantao database, we examined potential functional pathways based on the top 300 genes. GO and KEGG enrichment analysis revealed that CENPN plays important roles in the cell cycle, DNA replication, chromosome separation, and nuclear division, as well as several other key signaling pathways (Figure 2B).

Correlation between immune cell infiltration and CENPN expression

Immune cells that infiltrate tumors play a significant role in the tumor microenvironment and is linked to the development, spread, and metastasis of cancer[20, 21]. Therefore, we examined the connection between CENPN expression in STAD and the degree of immune cell infiltration (Figure 3A). A favorable correlation

was determined between CENPN expression and Th2 cells and NK CD56dim cells (Figure 3B,C). Mast cells , pDC ,NK cells ,and B cells were negatively correlated with the expression of CENPN (Figure 3D-G).

Effect of CENPN on STAD cell proliferation

To better understand the regulatory function of CENPN on the proliferation of STAD cells, we transiently transfected siRNAs to downregulate CENPN expression in AGS cell lines. The transfection effect was confirmed using western blotting. The CENPN expression in AGS cells in the transfection group was significantly lower than that in the control group (Figure 4A, $P < 0.01$). We then determined the proliferation and growth of AGS cells after CENPN downregulation using the CCK-8 assay. The results showed that CENPN downregulation significantly reduced the proliferation of AGS cells compared to the control group (Figure 4B, $P < 0.05$). To determine the effect of CENPN on STAD cell proliferation, an EdU test was also performed. The findings demonstrated that CENPN siRNA-transfected AGS cells had considerably lower proliferation ratios than the control group (Figure 4C, $P < 0.05$). In conclusion, the downregulation of CENP may inhibit the proliferation of STAD cells.

Effect of CENPN on the cell cycle of STAD cells

To further investigate the mechanism by which CENPN regulates the proliferation of human AGS cells, flow cytometry was used to detect cell cycle changes. CENPN knockdown in AGS cells resulted in an increased percentage of G0-G1 cells compared to the controls, while the percentage of cells in the S and G2-M phases decreased significantly (Figure 5,$P < 0.05$). These findings imply that CENPN may promote cell proliferation by regulating the cell cycle.

Effect of CENPN on STAD cell apoptosis

We used flow cytometry to investigate the role of CENPN in cell apoptosis in STAD. The percentages of early, late, and complete apoptosis in AGS cells were 23.67 % (12.7% in the control group), 7.39 % (2.98% in the control group), and 31.06 % (15.68% in the control group), respectively. After CENPN knockdown,the apoptosis rate of AGS cells was considerably higher than that of the control group (Figure 6,$P < 0.05$). Therefore, we concluded that CENPN knockdown promotes apoptosis in STAD cells.

Discussion

CENPN encoded proteins form nucleosome-associated complexes that are important for centromere assembly. CENPN binds to centromeres during the S and G2 phases and recruits other centromeric proteins[22]. Accurate chromosome segregation during mitosis is required for the proper transfer of genetic material from the mother to daughter cells. Missegregation of chromosomes can result in aneuploidy, a hallmark of cancer[8]. In humans, the histone H3 variant centromere protein A (CENPA) is present in the Functional centromeres. CENPA disruption or mutation results in failure of centromere formation[23, 24]. CENPN can recognize CENPA and regulate mitosis by forming the CENPA nucleosome-associated complex (NAC)[25, 26]. CENPN interacts with CENPA's centromere-targeting domain of CENPA to facilitate centromere assembly[27], and CENPN depletion leads to the loss of many other CENPs[25, 28]. Consequently, the CENPA nucleosome core region, which is necessary for centromere formation and chromosome segregation, is directly recognized by CENPN[29]. However, the mechanism of CENPN in STAD has not yet been reported, so it is of great significance to study the effect of CENPN in STAD.

TCGA and GTEx datasets were used in this study to evaluate the expression of CENPN in STAD. The evaluations revealed that STAD had higher CENPN expression than adjacent tumors ($P < 0.05$). We also examined CENPN expression in STAD paired samples from TCGA database. The results showed that STAD tissues had a greater level of CENPN expression than Matched normal tissues ($P < 0.001$). We verified this

finding using immunohistochemistry. This is consistent with the high expression of CENPN in malignancies of the lungs, liver, nasopharynx, and mouth[18, 19, 30, 31]. The ROC curve also supports CENPN's diagnostic value of CENPN. Additionally, we discovered that the degree of tumor invasion, TNM stage, and lymph node metastasis were substantially correlated with the level of CENPN expression in the tissues of patients with STAD, indicating that the abnormal expression of CENPN was intimately linked to the onset of STAD.

CENPN expression was found to be closely related to cell proliferation pathways such as"cell cycle," "DNA replication," "chromosome segregation," and "nuclear division" in our enrichment analysis. To validate this result, first we knocked down CENPN in AGS cells and discovered that depletion of CENPN significantly inhibited cell proliferation. Second, flow cytometry was used to detect changes in the cell cycle of AGS cells after CENPN knockdown. The results showed that knocked down of CENPN in AGS cells resulted in an increased percentage of G0-G1 cells ($P < 0.05$) and a significant decrease in the proportion of cells in the S and G2-M phases ($P < 0.05$). The S phase is the DNA synthesis phase that is critical for cell division and proliferation. These findings suggest that CENPN downregulation inhibits AGS cell proliferation. Studies have shown that CENPN knockdown promotes apoptosis in a variety of cancers[17, 19] . This study hypothesizes that CENPN might have the same effect in STAD. Flow cytometry was performed to investigate the relationship between CENPN expression and apoptosis in AGS cells. The results revealed that CENPN knockdown significantly increased the apoptotic rate of AGS cells.This suggested that CENPN is involved in apoptosis in STAD.

Immune cells play a crucial role in controlling the malignant behavior of tumor cells[32-34]. Immune cell infiltration has been linked to the development and spread of cancer[35, 36]. In this study, we explored the association between CENPN expression and immune cell infiltration in STAD. These findings demonstrate a positive correlation between Th2 cells and NK CD56dim cells and CENPN expression.Instead, it negatively correlated with mast cells,pDC,NK cells ,and B cells. These findings imply that the regulation of tumor immunity may be significantly influenced by CENPN.

However, this study has certain limitations. First, confounding variables may have skewed the data from open databases. Second, the sample numbers were limited in this study, and more cases are required to validate an observation. Finally, further research is needed to determine the precise mechanism of CENPN in STAD.

In conclusion, our investigation showed that CENPN was upregulated in STAD tissues and that the degree of tumor invasion, TNM stage, and lymph node metastasis were significantly correlated with CENPN expression. The proliferation of STAD cells was inhibited by depletion of CENPN, which also decreased the percentage of cells in S and G2-M phases and increased apoptosis. Immune cell infiltration and CENPN expression Significantly correlated. Therefore, we believe that CENPN could be a possible therapeutic target for STAD.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Ethics Statement

The current study was approved by the Ethics Committee of Shenyang Medical College and was performed in accordance with the Declaration of Helsinki,and written informed consent was received from the patient.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

Xiaojie Wang and Lan Luan designed the research. Xiaojie Wang, Keyuan Zhang and Cun Fu performed the research and collected the data. Xiaojie Wang, Fei Wu and Junjie Zhang analyzed the data. Xiaojie Wang drafted the manuscript. Bin Han and Hai Pan assessed all content. All authors reviewed the manuscript. Lan Luan made a final approval. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by grants from Shenyang Science and Technology Plan public health R &D special project under Grant 21-172-9-18.

Acknowledgments

We acknowledge Xiantao Academic for their assistance with bioinformatics analysis, statistics, and imaging.

References

1. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric Cancer. *Lancet* (2020) 396(10251):635-648. Epub 2020/08/31. doi: 10.1016/S0140-6736(20)31288-5.

2. Werner S, Chen H, Butt J, Michel A, Knebel P, Holleczek B, et al. Evaluation of the Diagnostic Value of 64 Simultaneously Measured Autoantibodies for Early Detection of Gastric Cancer. *Sci Rep*(2016) 6:25467. Epub 2016/05/04. doi: 10.1038/srep25467.

3. Kayamba V, Zyambo K, Kelly P. Presence of Blood in Gastric Juice: A Sensitive Marker for Gastric Cancer Screening in a Poor Resource Setting. *PLoS One* (2018) 13(10):e0205185. Epub 2018/10/16. doi: 10.1371/journal.pone.0205185.

4. Necula L, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, et al. Recent Advances in Gastric Cancer Early Diagnosis. *World J Gastroenterol* (2019) 25(17):2029-2044. Epub 2019/05/23. doi: 10.3748/wjg.v25.i17.2029.

5. Pentakota S, Zhou K, Smith C, Maffini S, Petrovic A, Morgan GP, et al. Decoding the Centromeric Nucleosome through Cenp-N. *Elife*(2017) 6. Epub 2017/12/28. doi: 10.7554/eLife.33442.

6. Schueler MG, Sullivan BA. Structural and Functional Dynamics of Human Centromeric Chromatin. *Annu Rev Genomics Hum Genet* (2006) 7:301-313. Epub 2006/06/08. doi: 10.1146/annurev.genom.7.080505.115613.

7. McKinley KL, Cheeseman IM. The Molecular Basis for Centromere Identity and Function. *Nat Rev Mol Cell Biol* (2016) 17(1):16-29. Epub 2015/11/26. doi: 10.1038/nrm.2015.5.

8. Kops GJ, Weaver BA, Cleveland DW. On the Road to Cancer: Aneuploidy and the Mitotic Checkpoint. *Nat Rev Cancer* (2005) 5(10):773-785. Epub 2005/10/01. doi: 10.1038/nrc1714.

9. Johnson SC, McClelland SE. Watching Cancer Cells Evolve through Chromosomal Instability. *Nature* (2019) 570(7760):166-167. Epub 2019/06/12. doi: 10.1038/d41586-019-01709-2.

10. Tanaka K, Hirota T. Chromosomal Instability: A Common Feature and a Therapeutic Target of Cancer. *Biochim Biophys Acta* (2016) 1866(1):64-75. Epub 2016/06/28. doi: 10.1016/j.bbcan.2016.06.002.

11. McAinsh AD, Meraldi P. The Ccan Complex: Linking Centromere Specification to Control of Kinetochore-Microtubule Dynamics.*Semin Cell Dev Biol* (2011) 22(9):946-952. Epub 2011/10/27. doi: 10.1016/j.semcdb.2011.09.016.

12. Yan K, Yang J, Zhang Z, McLaughlin SH, Chang L, Fasci D, et al. Structure of the Inner Kinetochore Ccan Complex Assembled onto a Centromeric Nucleosome. *Nature* (2019) 574(7777):278-282. Epub 2019/10/04. doi: 10.1038/s41586-019-1609-1.

13. Friedman S, Freitag M. Evolving Centromeres and Kinetochores.*Adv Genet* (2017) 98:1-41. Epub 2017/09/26. doi: 10.1016/bs.adgen.2017.07.001.

14. Freitag M. The Kinetochore Interaction Network (Kin) of Ascomycetes.*Mycologia* (2016) 108(3):485-505. Epub 2016/02/26. doi: 10.3852/15-182.

15. McKinley KL, Sekulic N, Guo LY, Tsinman T, Black BE, Cheeseman IM. The Cenp-L-N Complex Forms a Critical Node in an Integrated Meshwork of Interactions at the Centromere-Kinetochore Interface. *Mol Cell*(2015) 60(6):886-898. Epub 2015/12/25. doi: 10.1016/j.molcel.2015.10.027.

16. Mellone BG, Grive KJ, Shteyn V, Bowers SR, Oderberg I, Karpen GH. Assembly of Drosophila Centromeric Chromatin Proteins During Mitosis.*PLoS Genet* (2011) 7(5):e1002068. Epub 2011/05/19. doi: 10.1371/journal.pgen.1002068.

17. Wu H, Zhou Y, Wu H, Xu L, Yan Y, Tong X, et al. Cenpn Acts as a Novel Biomarker That Correlates with the Malignant Phenotypes of Glioma Cells. *Front Genet* (2021) 12:732376. Epub 2021/10/15. doi: 10.3389/fgene.2021.732376.

18. Wang Q, Yu X, Zheng Z, Chen F, Yang N, Zhou Y. Centromere Protein N May Be a Novel Malignant Prognostic Biomarker for Hepatocellular Carcinoma. *PeerJ* (2021) 9:e11342. Epub 2021/05/15. doi: 10.7717/peerj.11342.

19. Zheng Y, You H, Duan J, Chen B, Wu C, Chen P, et al. Centromere Protein N Promotes Lung Adenocarcinoma Progression by Activating Pi3k/Akt Signaling Pathway. *Genes Genomics* (2022) 44(9):1039-1049. Epub 2022/02/13. doi: 10.1007/s13258-021-01215-8.

20. Steven A, Seliger B. The Role of Immune Escape and Immune Cell Infiltration in Breast Cancer. *Breast Care (Basel)* (2018) 13(1):16-21. Epub 2018/06/29. doi: 10.1159/000486585.

21. Fridman WH, Galon J, Dieu-Nosjean MC, Cremer I, Fisson S, Damotte D, et al. Immune Infiltration in Human Cancer: Prognostic Significance and Disease Control. *Curr Top Microbiol Immunol* (2011) 344:1-24. Epub 2010/06/01. doi: 10.1007/82_2010_46.

22. Hellwig D, Emmerth S, Ulbricht T, Doring V, Hoischen C, Martin R, et al. Dynamics of Cenp-N Kinetochore Binding During the Cell Cycle.*J Cell Sci* (2011) 124(Pt 22):3871-3883. Epub 2011/11/22. doi: 10.1242/jcs.088625.

23. Cheeseman IM, Desai A. Molecular Architecture of the Kinetochore-Microtubule Interface. *Nat Rev Mol Cell Biol* (2008) 9(1):33-46. Epub 2007/12/22. doi: 10.1038/nrm2310.

24. Palmer DK, O'Day K, Trong HL, Charbonneau H, Margolis RL. Purification of the Centromere-Specific Protein Cenp-a and Demonstration That It Is a Distinctive Histone. *Proc Natl Acad Sci U S A* (1991) 88(9):3734-3738. Epub 1991/05/01. doi: 10.1073/pnas.88.9.3734.

25. Foltz DR, Jansen LE, Black BE, Bailey AO, Yates JR, 3rd, Cleveland DW. The Human Cenp-a Centromeric Nucleosome-Associated Complex.*Nat Cell Biol* (2006) 8(5):458-469. Epub 2006/04/20. doi: 10.1038/ncb1397.

26. Chittori S, Hong J, Saunders H, Feng H, Ghirlando R, Kelly AE, et al. Structural Mechanisms of Centromeric Nucleosome Recognition by the Kinetochore Protein Cenp-N. *Science* (2018) 359(6373):339-343. Epub 2017/12/23. doi: 10.1126/science.aar2781.

27. Tian T, Li X, Liu Y, Wang C, Liu X, Bi G, et al. Molecular Basis for Cenp-N Recognition of Cenp-a Nucleosome on the Human Kinetochore.*Cell Res* (2018) 28(3):374-378. Epub 2018/01/20. doi: 10.1038/cr.2018.13.

28. Carroll CW, Silva MC, Godek KM, Jansen LE, Straight AF. Centromere Assembly Requires the Direct Recognition of Cenp-a Nucleosomes by Cenp-N. *Nat Cell Biol* (2009) 11(7):896-902. Epub 2009/06/23. doi: 10.1038/ncb1899.

29. Carroll CW, Milks KJ, Straight AF. Dual Recognition of Cenp-a Nucleosomes Is Required for Centromere Assembly. *J Cell Biol*(2010) 189(7):1143-1155. Epub 2010/06/23. doi: 10.1083/jcb.201001013.

30. Qi CL, Huang ML, Zou Y, Yang R, Jiang Y, Sheng JF, et al. The Irf2/Cenp-N/Akt Signaling Axis Promotes Proliferation, Cell Cycling and Apoptosis Resistance in Nasopharyngeal Carcinoma Cells by Increasing Aerobic Glycolysis. *J Exp Clin Cancer Res* (2021) 40(1):390. Epub 2021/12/12. doi: 10.1186/s13046-021-02191-3.

31. Oka N, Kasamatsu A, Endo-Sakamoto Y, Eizuka K, Wagai S, Koide-Ishida N, et al. Centromere Protein N Participates in Cellular Proliferation of Human Oral Cancer by Cell-Cycle Enhancement. *J Cancer* (2019) 10(16):3728-3734. Epub 2019/07/25. doi: 10.7150/jca.32281.

32. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the Tumor Immune Microenvironment (Time) for Effective Therapy. *Nat Med* (2018) 24(5):541-550. Epub 2018/04/25. doi: 10.1038/s41591-018-0014-x.

33. Sahin Ozkan H, Ugurlu MU, Yumuk PF, Kaya H. Prognostic Role of Immune Markers in Triple Negative Breast Carcinoma. *Pathol Oncol Res* (2020) 26(4):2733-2745. Epub 2020/07/19. doi: 10.1007/s12253-020-00874-4.

34. Vilarino N, Bruna J, Bosch-Barrera J, Valiente M, Nadal E. Immunotherapy in Nsclc Patients with Brain Metastases. Understanding Brain Tumor Microenvironment and Dissecting Outcomes from Immune Checkpoint Blockade in the Clinic. *Cancer Treat Rev* (2020) 89:102067. Epub 2020/07/19. doi: 10.1016/j.ctrv.2020.102067.

35. Curigliano G. Gyneco-Oncological Genomics and Emerging Biomarkers for Cancer Treatment with Immune-Checkpoint Inhibitors. *Semin Cancer Biol* (2018) 52(Pt 2):253-258. Epub 2018/05/19. doi: 10.1016/j.semcancer.2018.05.004.

36. Sun R, Limkin EJ, Vakalopoulou M, Dercle L, Champiat S, Han SR, et al. A Radiomics Approach to Assess Tumour-Infiltrating Cd8 Cells and Response to Anti-Pd-1 or Anti-Pd-L1 Immunotherapy: An Imaging Biomarker, Retrospective Multicohort Study. *Lancet Oncol* (2018) 19(9):1180-1191. Epub 2018/08/19. doi: 10.1016/S1470-2045(18)30413-3.

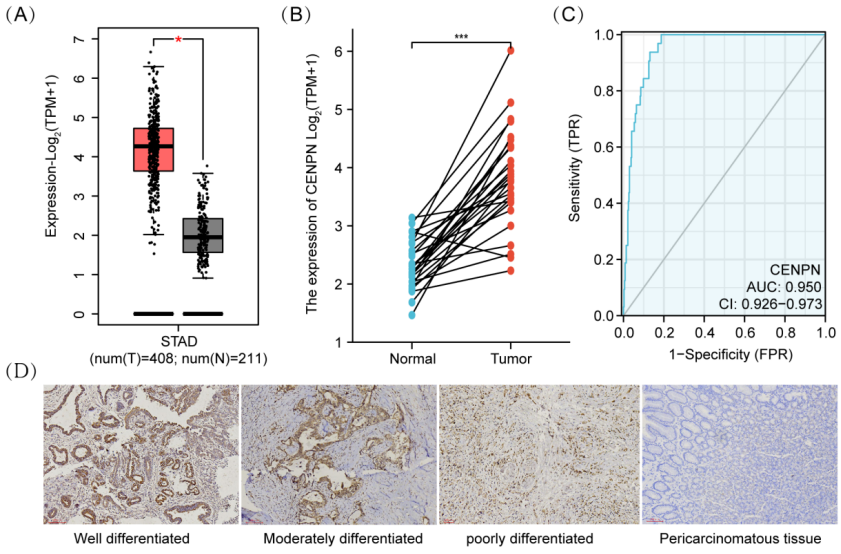

Figure 1: CENPN expression analysis in STAD. (A) CENPN expression in tumor and normal tissues in STAD data from TCGA and GTEx. (B) CENPN expression in paired tumor and normal tissues in STAD from TCGA. (C) ROC curves for classifying gastric adenocarcinoma versus normal gastric tissues in the TCGA database. TCGA, The Cancer Genome Atlas; GTEx, Genotype Tissue Expression Project; ROC, receiver operating characteristic.(D)Representative images of CENPN expression in gastric adenocarcinoma tissues and their matched paracancerous tissues. Data were shown as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$.

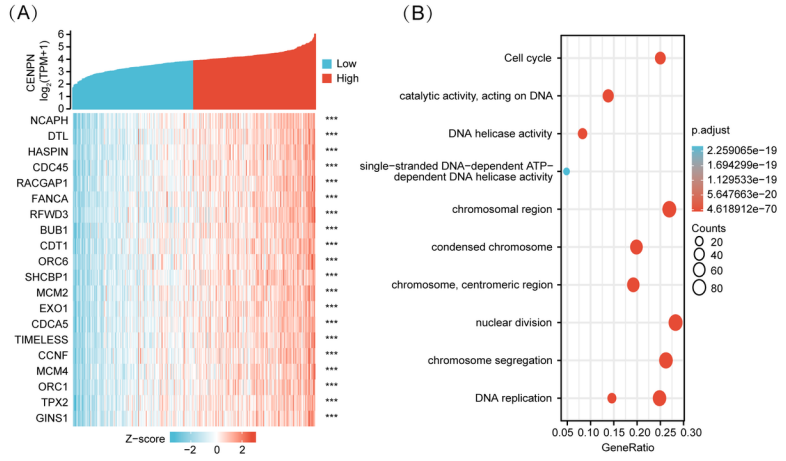

Figure 2: STAD co-expression gene screening and functional enrichment analysis of CENPN. (A) The heat map displays the top 20 genes most strongly associated with CENPN. (B) pathway analysis of GO|KEGG of the top 300 genes most positively related to CENPN. Data were shown as mean ± SD. $^{***}P < 0.001$.

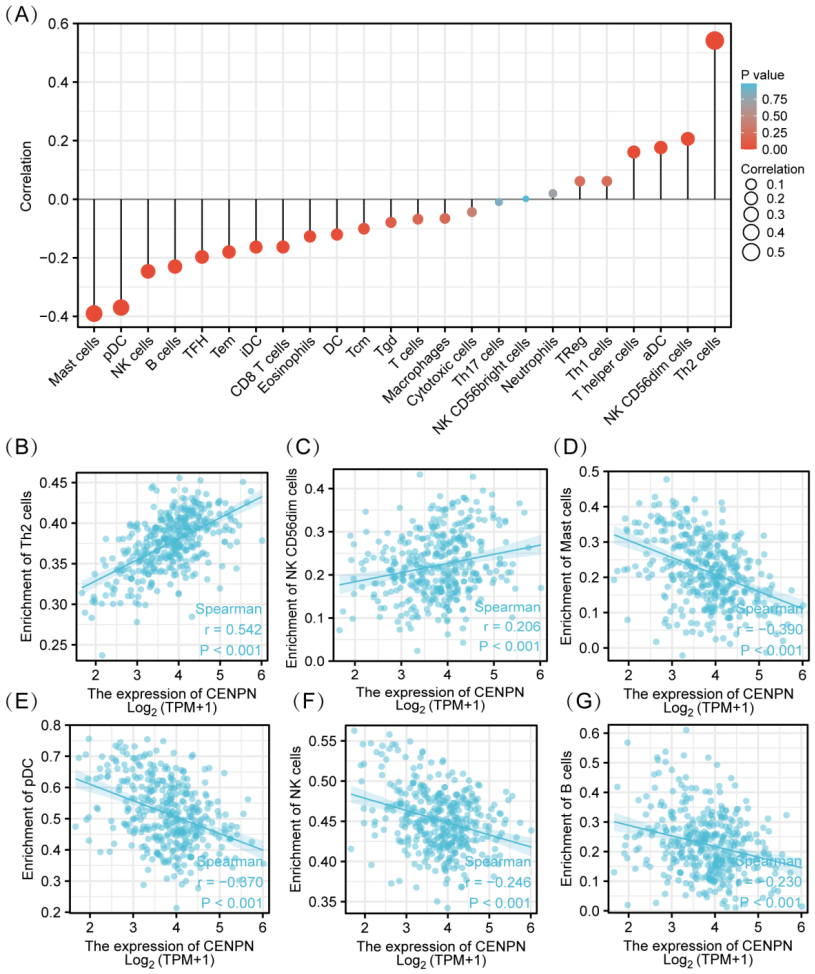

Figure 3: Correlation of CENPN expression with immune infiltration level in STAD. (A) Correlation between CENPN expression and relative abundance of 24 types of immune cell.The size of dot corresponds to the absolute Spearman's correlation coefficient values. (B–G) Correlation of CENPN expression with infiltration levels of Th2 cells , NK CD56dim cells,Mast cells , pDC cells ,NK cells, and B cells in STAD.Th2 cells,T-helper type 2 (Th2) cells;NK CD56dim cells,Natural killer CD56dim cells;pDC cells,plasmacytoid dendritic cells;NK cells, natural killer cells.

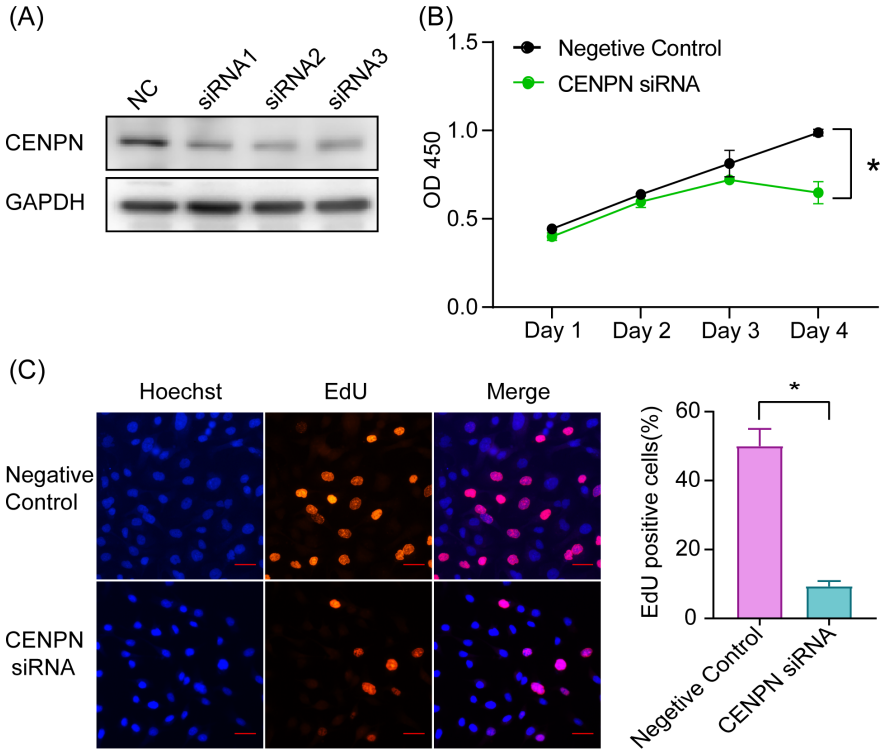

Figure 4: Effects of CENPN knockdown on the proliferation of STAD cells. (A) Expression of CENPN detected after transfection with siRNA by Western blot. (B,C) The proliferation of STAD cells was examined by CCK-8 assay (B) and EdU assay (C). Data were shown as mean ± SD. $^*P < 0.05$.

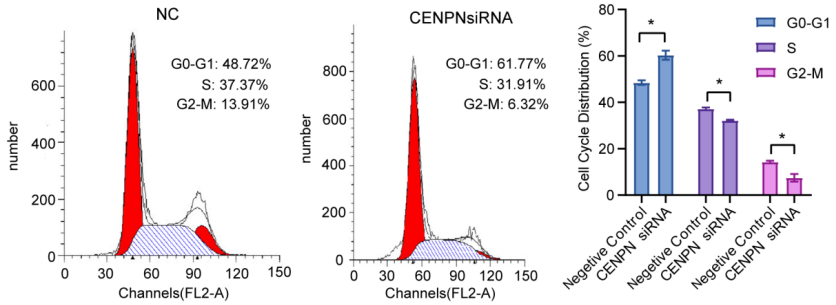

Figure 5: Flow cytometry assays were conducted to analyze the effect of CENPN downregulation on the cell cycle in AGS cells. Data were shown as mean ± SD. $^*P < 0.05$.

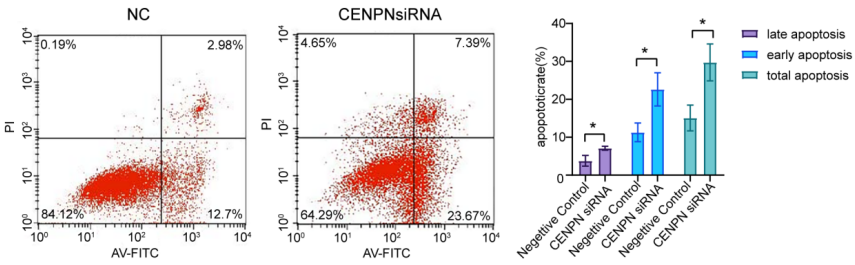

Figure 6: Flow cytometry assays were conducted to analyze the effect of CENPN downregulation on cell apoptosis in AGS cells. Data were shown as mean ± SD. $^*P < 0.05$.

Table 1 CENPN expression in STAD and adjacent tissues

Tissue type	Number of patients	CENPN negative(%)	CENPN positive(%)	χ^2value	P value
Gastric carcinoma	76	18(23.7)	58(76.3)	51.089	<0.01
Paracancerous tissue	76	62(81.6)	14(18.4)		

Table 2 The association of CENPN expression with clinicopathological characteristics in STAD

Characteristics	Number of patients	CENPN negative(%)	CENPN positive(%)	χ^2value	P value
Gender				0.723	0.395
Male	53	14(26.4)	39(73.6)		
Female	23	4(17.4)	19(82.6)		
Age				0.158	0.691
<65(years)	24	5(20.8)	19(79.2)		?¿?
65(years)	52	13(25.0)	39(75.0)		
Tumor size(cm)				1.653	0.198
<6	45	13(28.9)	32(71.1)		?¿?
6	31	5(16.1)	26(83.9)		
Differentiation				0.487	0.485
Well-Moderate	41	11(26.8)	30(73.2)		
poor	35	7(20.0)	28(80.0)		
Depth of invasion				8.507	0.004
T1+T2	25	11(44.0)	14(56.0)		
T3+T4	51	7(13.7)	44(86.3)		
TNM stage				7.991	0.005
I+II	37	14(37.8)	23(62.2)		
III+IV	39	4(10.3)	35(89.7)		
Nodal status				4.353	0.037
N0	23	9(39.1)	14(60.9)		
N1 N2 N3	53	9(17.0)	44(83.0)		

Hosted file

`Figure1.ai` available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

`Figure2.ai` available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

`Figure3.ai` available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

Figure4.ai available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

Figure5.ai available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

Figure6.ai available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

Table1.docx available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma

Hosted file

Table 2.docx available at https://authorea.com/users/517626/articles/592092-high-expression-of-centromere-protein-n-as-novel-biomarkers-for-gastric-adenocarcinoma