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ORIGINAL ARTICLE

Super enhancer-associated CCAT1 lncRNA promotes cell proliferation, invasion, migration, and epithelial-to-mesenchymal transition in gastric cancer

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Summary

Purpose: *Long non-coding RNAs (lncRNAs) promote gastric cancer invasion and metastasis via epithelial-to-mesenchymal transition (EMT). Super enhancers (SEs) regulate the expression of key oncogenic lncRNAs. Here, we attempted to identify EMT-regulating SE-associated lncRNAs.*

Methods: *H3K27ac histone acetylation was used as an SE marker, and chromatin immunoprecipitation sequencing (ChIP-seq) was performed to compare the SE landscape in SGC-7901 gastric adenocarcinoma and GES-1 in non-cancer gastric mucosa cell lines. Cancer associated transcript-1 (CCAT1) was knocked down in MKN-45 gastric adenocarcinoma cells and overexpressed in SGC-7901 cells. CCAT1 was detected by real-time PCR in GES-1, SGC-7901, and MKN-45 cells. Cell proliferation, migration and invasion were analyzed by Cell Counting kit-8, wound healing, and transwell assays, respectively. E-cadherin and N-cadherin expression was evaluated by western blotting.*

Results: *We identified 838 SEs in SGC-7901 and 206 SEs in GES-1 cells. SE-associated CCAT1 was identified as an EMT-related lncRNA upregulated in SGC-7901 and MKN-45 cells. Analysis of the Cancer Cell Line Encyclopedia and The Cancer Genome Atlas revealed elevated CCAT1 expression in various tumor cells and tissues. CCAT1 expression was associated with high cell proliferation, invasion, and migration and correlated negatively with E-cadherin, but positively with N-cadherin levels.*

Conclusions: *CCAT1 is upregulated in gastric cancer cells. CCAT1 is an EMT-promoting and SE-associated lncRNA that promotes cell proliferation, invasion, and migration. Therefore, CCAT1 may represent a potential diagnostic and therapeutic target for gastric cancer.*

Key words: *colon cancer associated transcript-1, epithelial-to-mesenchymal transition, gastric cancer, long non-coding RNA, super enhancer*

Introduction

Invasion and metastasis of cancer cells are the primary causes of death in gastric cancer patients. In recent years, epithelial-to-mesenchymal transition (EMT) has been found to be involved in the metastasis of cancer cells from the primary site to the target organ for colonization [1]. Exploring the key players in EMT will help clarify the mechanism of invasion and metastasis of gastric cancer cells and provide an important basis for the prevention and treatment of this disease.

During EMT, epithelial cells gradually lose adherence junctions and apical-basal polarity, gain migration and invasion capabilities, and transform into mesenchymal cells [2]. EMT involves multi-

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ple signaling pathways, such as Wnt/β-catenin, TGF-β/Smad, P13K/AKT, and Notch. During EMT, epithelial phenotype markers, including E-cadherin, laminin 1, ZO-1, desmoplakin, cytokeratin, and collagen IV, are downregulated [3], whereas mesenchymal phenotype markers, including N-cadherin, alpha-smooth muscle actin, vimentin, β-catenin, and fibronectin, as well as transcription factors such as SNAIL, TWIST1, SLUG, NF-κB, ZEB, and PRRX1, are upregulated [3-5].

The EMT process is regulated by transcription factors, microRNAs/miRs, long non-coding RNAs (lncRNAs), circular RNAs, and selective splicing factors [6]. Numerous lncRNAs have been found to promote cancer cell invasion and metastasis by inducing EMT [7,8]. Importantly, lncRNAs respond faster than transcription factors because they do not require protein translation [9]. Multiple lncRNAs induce EMT in gastric cancer cells and thus promote their metastasis. LncRNA expression is regulated by transcription factors, histone modifications, DNA methylation, enhancers, and super enhancers (SEs) [10-13]. Enhancers are the main determinants of cell morphology and function, whereas SEs exert a four-fold stronger transcriptional regulatory effect on target genes compared to a typical enhancer [14].

In 2013, Whyte and co-workers proposed a SE model based on a typical enhancer. SEs are considerably long (8-20 kb) cis-acting elements that promote transcription. SEs bind strongly to master transcription factors and cofactors, whose main function is to regulate the expression of genes, including oncogenes that control cell identity [14]. SEs are large clusters composed of multiple enhancers and rely on common epigenetic markers, such as methylation or acetylation of histone H3. For example, H3K4me1, H3K27me3, and H3K27ac define the momentum, preparation and activation of SEs, respectively [15,16].

SEs can promote the malignant transformation of non-cancer cells into cancer cells [17]. Moreover, during tumorigenesis, cancer cells can concentrate SEs near oncogenes to regulate their expression [18]. Overexpression of these SE-related genes plays an important role in tumor pathogenesis. Among them, SE-lncRNAs have received increasing attention [19]. In 2018 Xie et al discovered that SEs promoted the expression of LINC01503 lncRNA in squamous cell carcinoma (SCC), and its overexpression stimulated proliferation, migration and invasion of SCC cells, as well as growth of subcutaneous transplanted tumors in nude mice [1]. In 2019 Peng et al found that SE-associated HCCL5 lncRNA promoted EMT in HCC cells by upregulating the expression of EMT-related transcription factors, including SNAIL, SLUG, ZEB1, and TWIST1 [20]. However, it remains unclear whether SE-lncRNAs are involved in the invasion and metastasis of gastric cancer.

In the present study, SEs in gastric cancer and healthy gastric mucosa cells were detected by H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq). Differences were then compared to identify gastric cancer-related SEs. Genes related to these SEs were predicted by bioinformatics tools and compared to the lncRNAs obtained previously by microarray analysis of EMT-related lncRNAs. We report that the EMT-related colon cancer associated transcript-1 (CCAT1) lncRNA is regulated by SEs in gastric cancer.

Methods

Cell lines and cell culture

GES-1 is a human cell line derived from healthy fetal gastric mucosal epithelial cells. SGC-7901 and MKN-45 are human gastric adenocarcinoma cell lines. All three cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were cultured in RPMI 1640 complete medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and incubated at 37°C with 5% CO_2.

Cell transfection

The small interfering RNA (siRNA) targeting CCAT1 and the plasmid pCDNA3.1-CCAT1 were purchased from Hanbio (Shanghai, China). The CCAT1 siRNA sequence was 5'-CGGCAGGCATTAGAGATGAACAGCA-3', while that of scramble siRNA was 5'-CCUACGCCACCAAUUUCGU-3'. Cells were transfected with siRNAs and plasmids using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected 48 h post-transfection.

Growth curve

A cell suspension (100 μL) was inoculated in 96-well plates, and the plates were placed in an incubator at 37°C with 5%CO_2 saturated humidity for 24 h. Cell Counting kit-8 solution (10 μL) was added to each well at 24, 48, and 72 h, and the plate was incubated for 1-4 h. Absorbance at 450 nm was measured with a microplate reader and was used to plot cell proliferation curves.

Transwell assays

Transwell assays were performed in 24-well Transwell plates with an 8-mm aperture. Prior to the experiment, the bottom of the Transwell chambers was covered with Matrigel and incubated at 37°C for 30 min. In the experiment, 600 μL medium containing 15% FBS was added to the bottom chambers, and 1×10^4 cells in 300 μL medium containing 10% FBS were added to the top chambers. The cells were cultured in an incubator at 37°C and 5%CO_2 for another 36 h. The Matrigel and cells in the upper chambers were wiped off with cotton swabs.

The Transwell was removed and stained with crystal violet dye. Cells were counted in ten randomly selected fields of view.

RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The concentration and purity of the extracted total RNA were determined using a UV spectrophotometer. Total RNA was reverse-transcribed using the PrimeScript RT Reagent kit (TaKaRa Bio, Beijing, China). Real-time quantitative PCR (RT-qPCR) was performed in the CFX96™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Premix Ex Taq™ II (TaKaRa Bio). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. A comparative CT method was used for quantitative detection, and a standard curve was plotted.

Chromatin immunoprecipitation sequencing analysis

ChIP experiments were performed using the Epi™ Chromatin Immunoprecipitation kit (R1802; Epibiotek, Guangzhou, China). Briefly, when GES-1 or SGC-7901 cells reached 80% confluence, they were collected, cross-linked with 1% formaldehyde for 10 min, and quenched with 0.125 M glycine for 5 min. Cells were lysed in 1 mL lysis buffer and incubated under rotation for 30 min at 4°C. Cell lysates were centrifuged at 2,400×g for 10 min at 4°C to isolate the nuclei. Nuclei were resuspended in digestion buffer and subjected to enzymatic shearing to obtain chromatin fragments with an average size of 200-500 bp. The reaction was carried out at 37°C for 10-15 min, stopped, and the fragmented chromatin was centrifuged at 18,000×g for 10 min at 4°C. The supernatant was transferred to a new 1.5-mL tube (Eppendorf, Hamburg, Germany), and the ChIP reaction mix containing protein A/G magnetic beads, ChIP IP buffer, antibody against H3K27ac, and protease inhibitor cocktail was rotated at 4°C overnight. The next day, the protein A/G magnetic beads were washed, after which chromatin was eluted in reverse cross-linking buffer and incubated at 65°C for 3 h. The ChIP DNA was then treated with RNase A and protease K at 37°C for 30 min and purified using phenol chloroform. ChIP DNA was processed for library generation using the QIAseq Ultralow Input Library Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

Bioinformatics analysis of ChIP-seq data

ChIP-seq data analysis was performed as described previously [17]. Briefly, raw reads were filtered and trimmed to remove sequencing adaptors and low-quality reads. After quality control, clean reads were mapped to the human genome (version hg38 in Bowtie 2 2.5.1) [21]. Only uniquely mapped reads were retained. Peaks were assigned using MACS version 1.4.2. Predicted enhancers were determined as peak regions of at least 2kb from the annotated transcription start site (TSS). Constitutive enhancers that occurred within 12.5 kb were further stitched together using the ROSE algorithm version 2 to identify SEs. All enhancer regions, including stitched and solo enhancers, were plotted in increasing order based on their ChIP-seq signals. Enhancers above the inflection point of the curve were defined as SEs. SEs were assigned to genes with transcription start site flanking a 500-bp window of the SEs, and these genes were defined as SE-associated genes.

To evaluate and predict SE function, SE-associated genes were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis using the DAVID annotation tool (https://david.ncifcrf.gov/).

Western blotting

Western blotting was used to detect E-cadherin and N-cadherin levels. Cells were lysed in RIPA buffer to extract proteins. Protein concentration was measured using a BCA protein assay kit, according to the manufacturer's instructions. The protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were incubated with the primary antibody overnight in a cold room and with the secondary antibody for 2 h at room temperature. An enhanced chemiluminescence kit was used to visualize antibody binding. The density of protein bands was normalized to that of GAPDH using a mouse anti-GAPDH monoclonal antibody. The results were normalized to control values.

Wound healing assays

Cells in logarithmic growth phase were digested and counted, and cell suspensions at 1×10^5 cells/mL were prepared. An aliquot (1 mL) was added to each well of the 6-well culture plates, and the cells were cultured in an incubator at 37°C with 5%CO_2 until they formed a monolayer. Wounds were created in the cell monolayer with a 200-μL pipette tip, the culture medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) three times. Wound closure of cell cultures at 0, 12, and 24 h was observed and recorded.

Statistics

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as mean±standard deviation. Comparisons between groups were performed using two-tailed paired Student's *t*-test, chi square test, or one-way analysis of variance (ANOVA). All tests were two-sided. $P<0.05$ was considered statistically significant.

Results

Super enhancer landscape in gastric cancer and healthy gastric mucosa cells

H3K27ac was employed as SE marker, and ChIP-seq was performed using an antibody that recognized H3K27ac modifications to compare SE distribution in gastric cancer versus non-tumor gastric mucosa cells. The H3K27ac ChIP-seq

data of both cell types were analyzed to identify H3K27ac enrichment regions (peaks). To identify gastric cancer-specific SEs, H3K27ac signals from sliding windows of 12.5-kb genomic regions containing enhancers were ranked. SEs were defined as presenting a four-fold higher H3K27ac ChIP-seq signal compared to the surrounding areas. A total of 838 SEs together with 768 SE-related genes were identified in gastric cancer cells, while 206 SEs and 190 SE-related genes were identified in non-cancer cells (Figure 1). To understand the biological features of SEs, we performed GO and KEGG analyses of SE-associated genes. In GO analysis, tumor-related biological processes were enriched in gastric cancer cell lines compared to non-cancer cells (Figure 2A,B). In the latter, most SE-associated genes participated in cell metabolism and development. Similarly, KEGG analysis revealed critical pathways in gastric cancer cells and non-cancer cells, with tumor-related pathways enriched in the former (Figure 2C,D).

Identification of SE-lncRNAs associated with the EMT pathway in gastric cancer

Previously, to identify EMT-related lncRNAs in gastric cancer, we utilized the LncPath™ Human EMT Pathway LncRNA Microarray on three gastric cancer tissues and their paired adjacent non-neoplastic gastric tissues. A total of 390 differentially expressed lncRNAs were identified, of which 22 lncRNAs were upregulated and 17 were downregulated by more than 1.5 fold [21]. Here, CCAT1 lncRNA was related to both SEs and EMT in gastric cancer (Figure 3). Hence, we speculated that CCAT1 might promote the invasion and metastasis of gastric cancer cells by inducing the EMT pathway.

Characterization of CCAT1 expression in gastric cancer

To understand CCAT1 expression in gastric cancer cells, we first looked at H3K27ac occupancy of CCAT1 loci in SGC-7901 and GES-1 cells (Figure 4A). The Cancer Cell Line Encyclopedia (CCLE) data

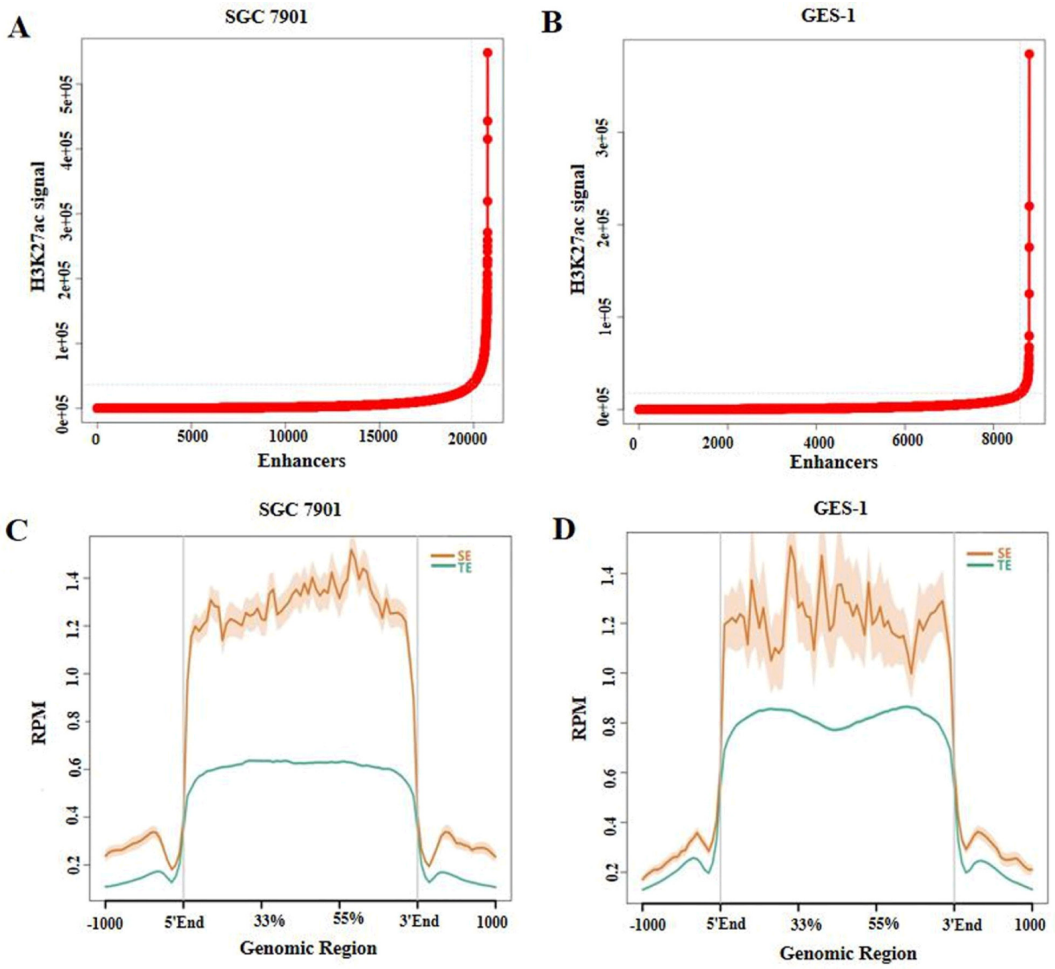

Figure 1. Super enhancer (SE) scatter plots and histograms of H3K27ac signal distribution in SGC-7901 and GES-1 cell lines. **A:** Scatter plots of SEs in SGC-7901 and GES-1 cells. All stitched regions were ranked by H3K27ac signal. Enhancers above the inflection point of the curve are defined as SEs. **B:** Bimodal H3K27ac signal distribution at identified SE and typical enhancer (TE) regions.

showed that CCAT1 was highly expressed in various tumor cells, including gastric cancer cells (Figure 4B). CCAT1 expression appeared significantly higher in several types of tumor tissues, including gastric cancer, also when using the The Cancer Genome Atlas (TCGA) Gene Expression Profiling Interactive Analysis tool (Figure 4C). Finally, qRT-PCR confirmed significantly higher CCAT1 expression in SGC-7901 and MKN-45 cells than in GES-1 cells (Figure 4D).

CCAT1 promotes cell proliferation, invasion, migration, and EMT in gastric cancer

To investigate the biological significance of CCAT1 in gastric cancer cells, we applied loss-of-function and gain-of-function approaches. A CCAT1 expression vector was used to transfect SGC-7901 cells and overexpress CCAT1 mRNA, whereas CCAT1-siRNA was used to transfect MKN-45 cells and knock down CCAT1 mRNA expression (Figure

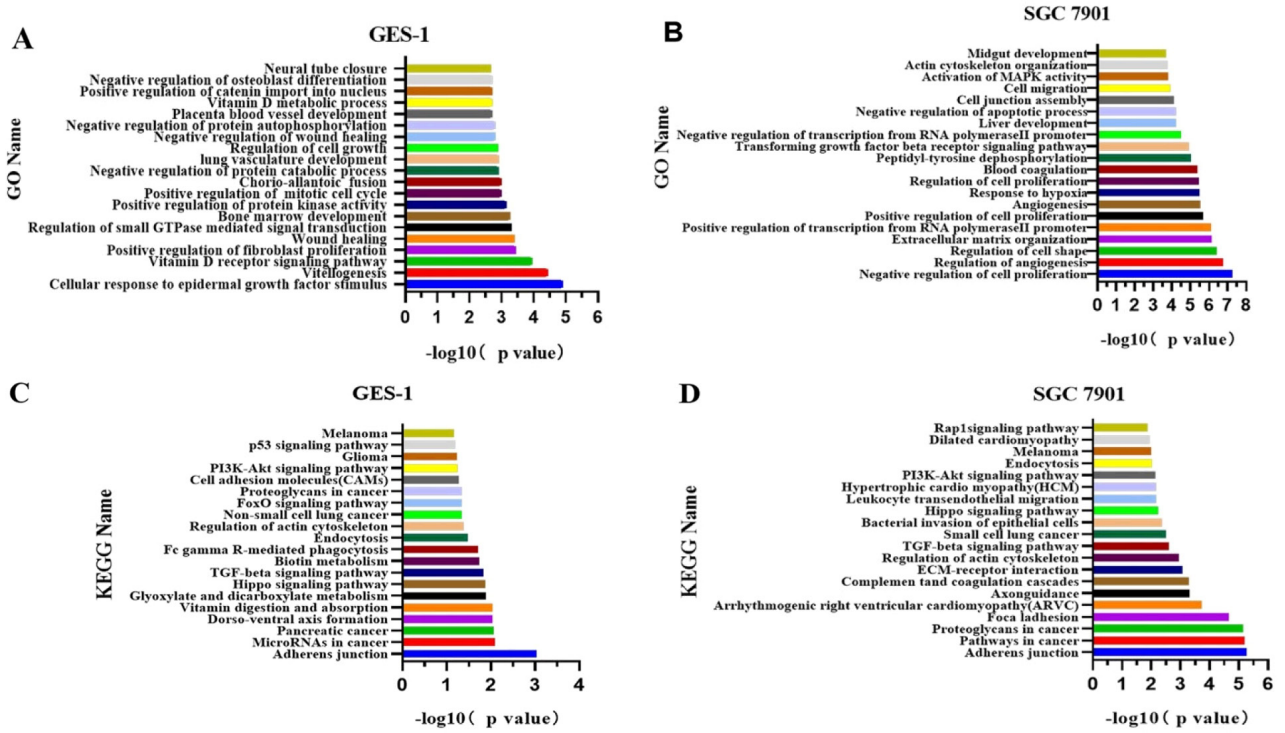

Figure 2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of super enhancer (SE)-associated genes in SGC-7901 and GES-1 cell lines. **A,B:** GO analysis for SE-associated genes in SGC-7901 and GES-1 cells. The top 20 GO enrichment processes are listed. **C,D:** KEGG analysis for SE-associated genes in SGC-7901 and GES-1 cells. The top 20 KEGG enrichment pathways are listed.

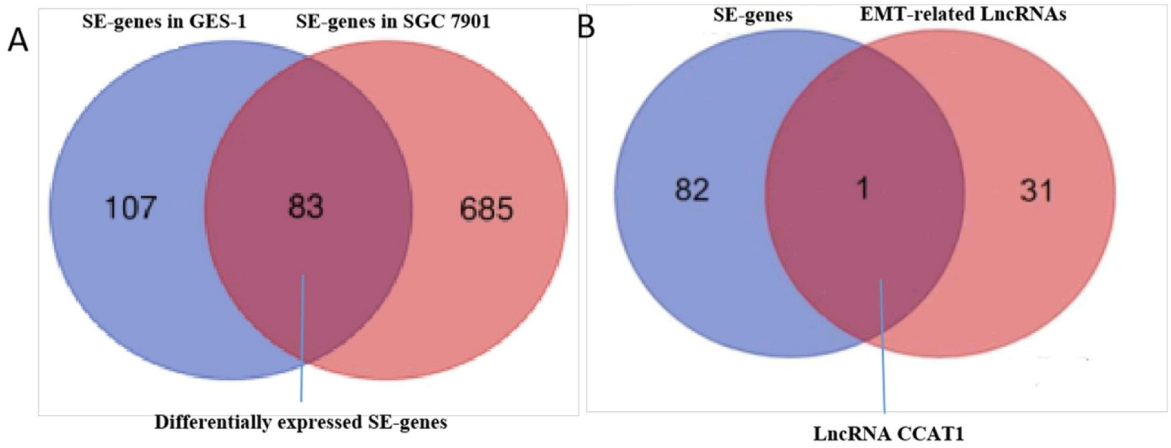

Figure 3. Identification of super enhancer (SE)-lncRNAs associated with epithelial-to-mesenchymal transition (EMT) in gastric cancer. **A:** Venn diagram showing differentially expressed SE genes in SGC-7901 and GES-1 cell lines. **B:** Venn diagram showing the link between the SE-lncRNA colon cancer associated transcript-1 (CCAT1) and EMT in gastric cancer.

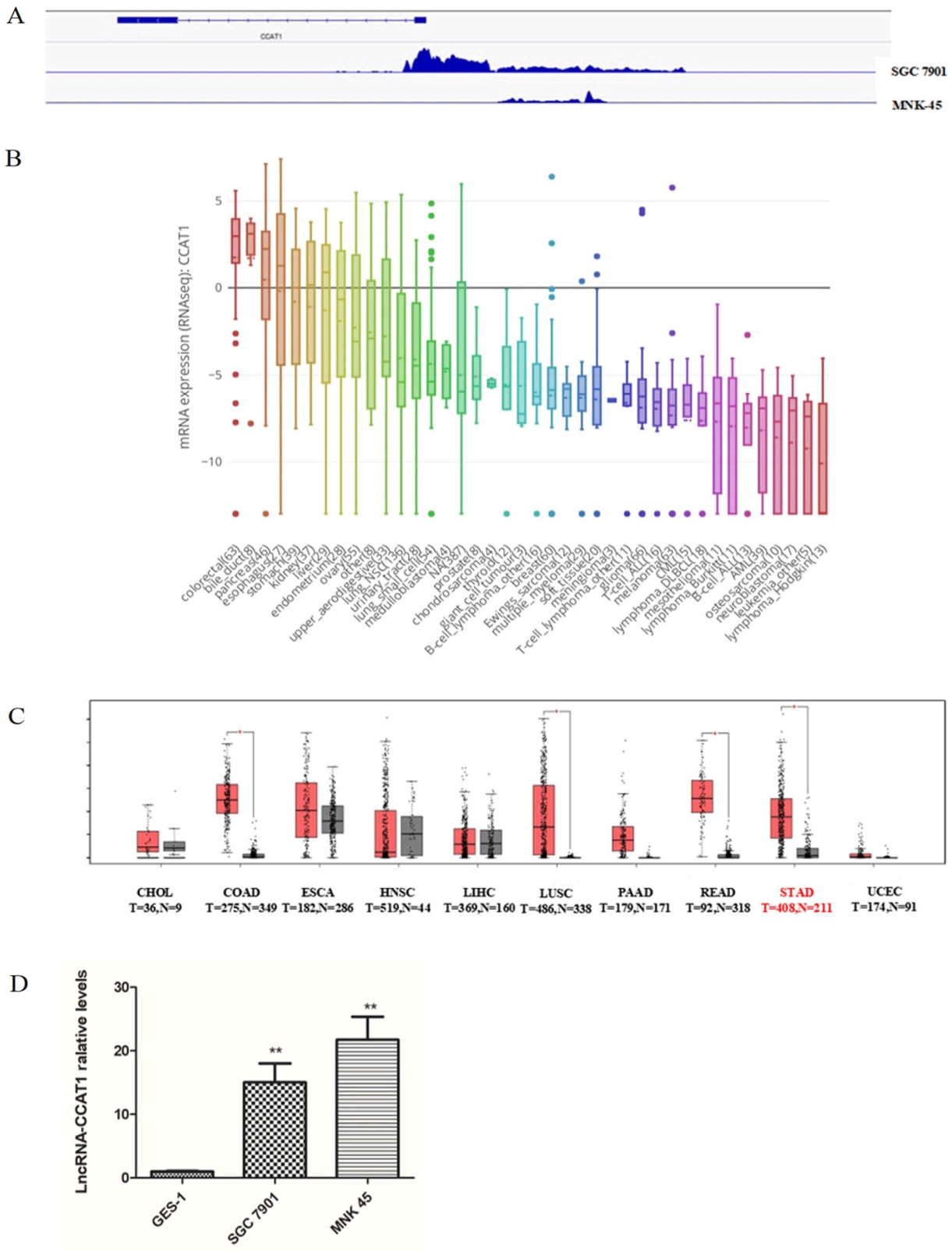

Figure 4. Characterization of colon cancer associated transcript-1 (CCAT1) expression in gastric cancer. **A:** Gene tracks of H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq) occupancy at CCAT1 loci in SGC-7901 and GES-1 cell lines. The X-axis shows genomic position, and the Y-axis shows the ChIP-seq occupancy signal in reads per million mapped reads per base pair (rpm/bp). **B:** CCAT1 expression in various cancer cells according to the Cancer Cell Line Encyclopedia. **C:** CCAT1 expression in several types of tumors according to The Cancer Genome Atlas; tumor tissues and matched healthy tissues were analyzed using the Gene Expression Profiling Interactive Analysis online database. Each point represents one tissue sample. **D:** CCAT1 mRNA levels in GES-1, SGC-7901, and MKN-45 cells as measured by RT-qPCR. *p<0.05 and **p<0.01.

5A). The proliferation efficiency of gastric carcinoma cells was evaluated with a Cell Counting kit-8 assay. Compared to the control, CCAT1 overexpression promoted the proliferation of SGC-7901 cells, while CCAT1 knock down inhibited that of MKN-45 cells (Figure 5B). Transwell and wound healing assays indicated that CCAT1 knockdown significantly inhibited invasion and migration in MKN-45 cells (Figure 5C,E). In contrast, CCAT1 overexpression promoted cell invasion and migration of SGC-7901 cells (Figure 5D,F). Finally, to evaluate the relationship between CCAT1 and EMT, we measured the expression of E-cadherin and N-cadherin by western blotting. CCAT1 expression correlated negatively with E-cadherin and positively with N-cadherin levels (Figure 5G). These results suggest that CCAT1 induces invasion and metastasis of gastric cancer cells via EMT.

Discussion

During tumor growth, single epithelial tumor cells are shed from the primary tumor site and undergo EMT. The newly formed mesenchymal cells are capable of migration, enter the blood stream where they become circulating tumor cells, and eventually extravasate into distant organs. At that point, they undergo mesenchymal-to-epithelial transition (MET) and form epithelial cells capable of proliferation, colonization of the target organ,

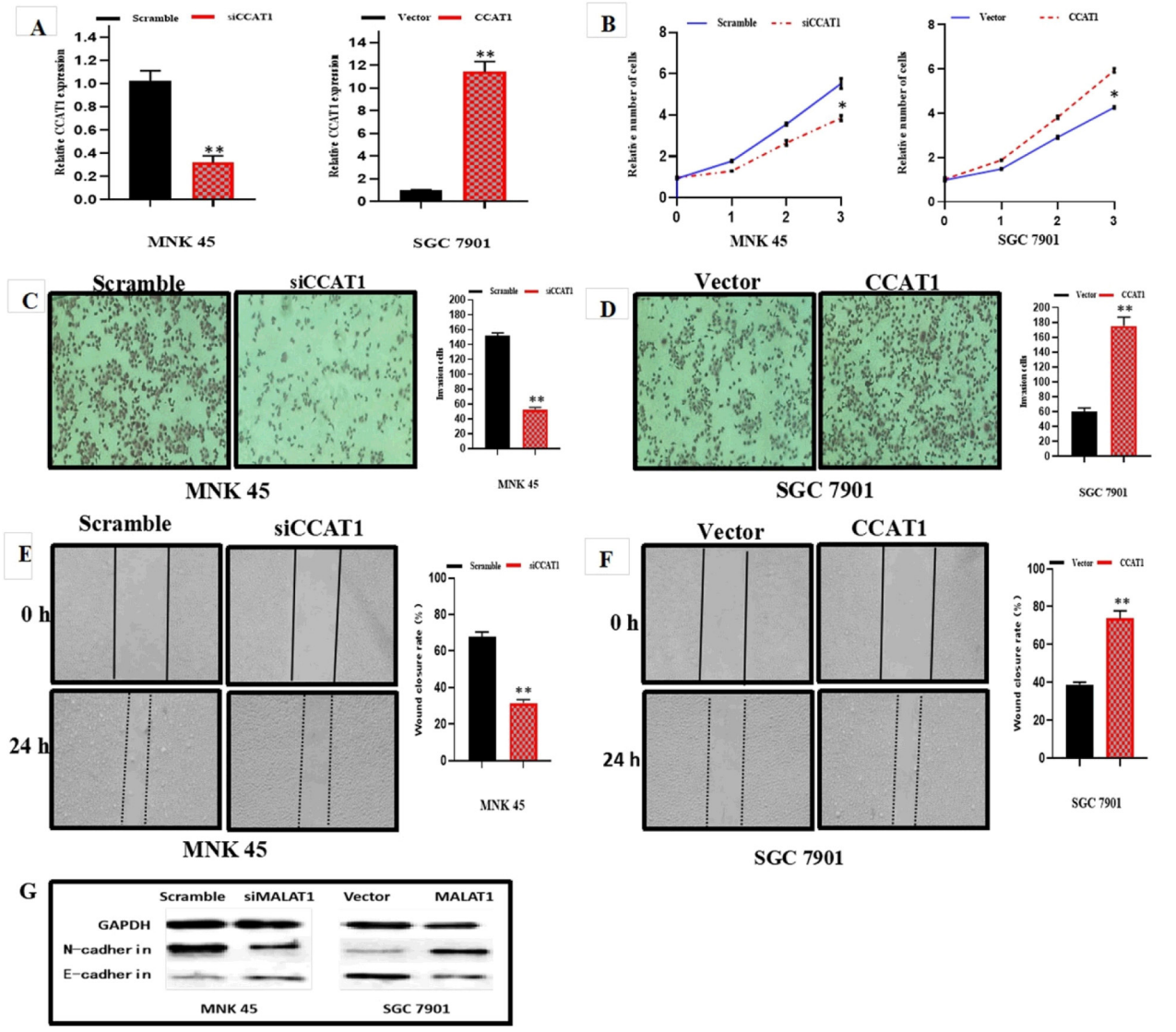

Figure 5. CCAT1 promotes the proliferation, invasion, migration, and endothelial-to-mesenchymal transition (EMT) of gastric cancer cells. **A:** RT-qPCR analysis validating the silencing (MKN-45 cells) or overexpression (SGC-7901 cells) of colon cancer associated transcript-1 (CCAT1). **B:** Cell Counting Kit-8 (CCK8) assays in gastric cancer cells upon CCAT1 knockdown or overexpression. **C-F:** Migration and invasion assays showing the effect of CCAT1 on these behaviors in gastric cancer cells. **G:** Western blot assay showing the effect of CCAT1 on E-cadherin and N-cadherin levels in gastric cancer cells. *p<0.05 and **p<0.01.

and metastasis [23,24]. Abnormal activation of EMT promotes the invasion and migration of tumor cells during metastasis, whereas MET is believed to promote the proliferation and growth of tumor cells after reaching the target organs [25].

Numerous lncRNAs promote gastric cancer metastasis by inducing EMT. In 2017 Chen et al found that MALAT1 expression was upregulated in gastric cancer cells, E-cadherin mRNA and protein levels were decreased, while vimentin expression was increased [26]. In 2017 Lee et al further confirmed that MALAT1 affected the EMT process and promoted tumor metastasis by regulating mesenchymal cell markers (e.g., SNAIL, E-cadherin, and ZEB1) [27]. HOTAIR lncRNA expression was found to be upregulated in gastric cancer cells. In such cells, E-cadherin expression was decreased, while those of N-cadherin and vimentin were increased. HOTAIR may promote gastric cancer metastasis by recruiting the histone methyltransferase PRC2 and binding to the miR-34a, which then activates the HGF/c-met/Snail pathway and stimulates EMT [28,29]. Furthermore, many lncRNAs, including FRLnc1, ATB, XIST, ZFAS1, and UCA1, as well as the large intergenic non-coding RNAs 00978 and 00152, can affect gastric cancer metastasis by promoting EMT [30]. In our previous study using the LncPath™ Human EMT Pathway LncRNA Microarray to compare EMT-related lncRNA expression profiles in gastric cancer and adjacent healthy tissues, we identified 390 differentially expressed lncRNAs [22]. This result suggests that lncRNAs play an important role in the EMT process in gastric cancer cells.

Given that lncRNA expression is regulated by multiple factors, including SEs [11,23,30], here, we compared the SE landscape in gastric cancer cells and healthy gastric mucosa cells by H3K27ac ChIP-seq. This approach allowed us to identify SE-associated oncogenes. GO and KEGG pathway analyses revealed that SE-associated genes were closely linked to tumor occurrence and development. Furthermore, combined with EMT-related lncRNA microarray analysis results, we discovered that the CCAT1 lncRNA was related to both SE and EMT in gastric cancer.

Human CCAT1 lncRNA encompasses 2,628 bp located at chr8q24.21. CCAT1 contains two exons and a poly-A tail and is primarily expressed in the nucleus. CCAT1 was originally found to be highly expressed in colon cancer [32], but its carcinogenic properties have been reported also in colorectal cancer [33], non-small-cell lung cancer [34], hepatocellular carcinoma [35], esophageal SCC [12], and gastric cancer [36].

In our study, CCAT1 expression was upregulated in gastric cancer cells, and CCAT1 overexpression promoted cell proliferation, migration and invasion in SGC-7901 and MKN-45 cells. These results were also confirmed by CCLE and TCGA data. Moreover, CCAT1 expression correlated negatively with E-cadherin and positively with N-cadherin levels, suggesting that CCAT1 induces EMT in gastric cancer cells. Many studies have shown that CCAT1 is closely related to EMT in colorectal cancer [37], glioma [38], intrahepatic bile duct cell carcinoma [39], ovarian cancer [40,41], lung cancer [42] and cervical cancer [43]. Combining the above results, we speculate that CCAT1 might promote the invasion and metastasis of gastric cancer cells by inducing EMT. In 2018 Jiang et al found that SCC master transcription factors TP63 and SOX2 could activate the transcription of CCAT1 by binding directly to its SE and promoter. In contrast, CCAT1 recruits TP63 and SOX2 to form RNA/protein complexes, which activate the epithelial growth factor receptor (EGFR) expression by binding to the SE of EGFR. These complex regulatory circuits elevate the expression of CCAT1 and EGFR and promote SCC development by activating the MEK/ERK1/2 and PI3K/AKT signaling pathways [43]. So far, no attempts have been made to understand the mechanism described in this study, by which SE regulates CCAT1.

In summary, we detected and compared the SE landscape in gastric cancer cells and healthy gastric mucosa cells and identified EMT-related lncRNAs in gastric cancer. We found that CCAT1 lncRNA acted both as SE-controlled element and EMT-related lncRNA in gastric cancer. CCAT1 was upregulated in gastric cancer cell lines, which was confirmed by CCLE and TCGA data. Also, CCAT1 promoted cell proliferation and migration of gastric cancer cells and correlated negatively with E-cadherin but positively with N-cadherin and we conclude that CCAT1 overexpression may promote invasion and metastasis of gastric cancer cells by inducing the EMT pathway.

Acknowledgements

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Conflict of interests

The authors declare no conflict of interests.

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