CHD8 is an independent prognostic indicator that regulates Wnt/β-catenin signaling and the cell cycle in gastric cancer

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Abstract. The chromodomain helicase DNA-binding (CHD) family comprises a class of chromatin remodeling enzymes. Previous studies suggest that *CHD8* may negatively regulate various genes and signaling pathways, such as the Wnt/β-catenin pathway. However, few studies have investigated the role of *CHD8* in cancer cells. We analyzed the expression of *CHD8* in cancer lesions and corresponding non-cancerous tissues to demonstrate the prognostic significance of *CHD8* expression in 101 cases of gastric cancer. We also investigated the functional implications of aberrant *CHD8* expression by conducting gene set enrichment analysis (GSEA). Expression of *CHD8* mRNA was significantly lower in gastric cancer tissues compared to that in corresponding normal tissues (P=0.003). In multivariate analysis for overall survival, we found that *CHD8* expression was an independent prognostic factor in gastric cancer. Moreover, GSEA revealed that *CHD8* was significantly associated with genes involved in the Wnt/β-catenin pathway and in the cell cycle. In addition, knockdown of *CHD8* expression in the gastric cancer cell lines, MKN45 and NUGC4, promoted proliferation. In conclusion, the present study suggests that loss of *CHD8* expression may be a novel indicator for biological aggressiveness in gastric cancer.

Introduction

Gastric cancer is one of the most common malignant tumors in the world. According to data from the National Cancer Institute (NCI), it is estimated that more than 24,000 patients are diagnosed with gastric cancer each year in the United States (1). Patients with advanced gastric cancer still face a poor prognosis. Identification of genes responsible for the development and progression of gastric cancer and a clear understanding of the clinical significance of these genes are both important for the diagnosis and adequate treatment of this disease.

Chromatin remodeling is a key mechanism regulating gene expression. The chromodomain helicase DNA-binding (CHD) family comprises a group of chromatin remodeling enzymes that use the energy from ATP hydrolysis to alter the structure or position of the nucleosome (2-5). In humans, 9 CHD family proteins have been identified to date (2,5). These proteins share common tandem chromodomains in the N-terminal region and an ATPase-helicase domain in the central region (2,5). Several studies have demonstrated that chromatin remodeling by CHD family members is involved in the pathogenesis of different types of cancers. For example, the *CHD7* gene is known to be mutated in small cell lung cancer (SCLC) tissues and SCLC cell lines express the *PVT1-CHD7* fusion gene (6). Moreover, CHD5 has been shown to control proliferation and apoptosis via the p19-p53 pathway, functioning as a tumor suppressor (7). In humans, *CHD5* is inactivated not only by deletion, but also by hypermethylation in several types of cancer (7).

CHD8 was originally isolated as a negative regulator of the Wnt/β-catenin pathway when it was found to suppress β-catenin function (8,9). CHD8 has also been suggested to regulate the expression of various genes, including *cyclin E2* and *HOXA2* (10-14). In contrast, CHD8 can also bind to p53 and suppress its transactivation activity by recruiting histone H1 during embryogenesis (15). However, the role of CHD8 in solid malignant tumors has not yet been elucidated.

In the present study, we analyzed *CHD8* mRNA expression using clinical samples from 101 patients diagnosed with primary gastric cancer. We then examined the relationship between *CHD8* mRNA expression and clinicopathological factors and determined the clinical significance of aberrant *CHD8* expression. Moreover, we investigated the functional role of CHD8 in gastric cancer by analyzing expression array data *in silico* and confirmed the biological significance of CHD8 in gastric cancer cells *in vitro*.

Materials and methods

Clinical samples and cell lines. A total of 101 gastric cancer patients were enrolled in this study. All patients underwent

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surgery without preoperative treatments such as chemotherapy and radiotherapy. Tumor and adjacent normal tissues were obtained. Total RNA was extracted using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol. Patients were closely observed each month after surgery and the mean postoperative follow-up period was 2.8 years. Histopathological evaluations were assessed according to the Japanese Classification of Gastric Cancer, 3rd English edition.

MKN-45 cell lines were provided by the American Type Culture Collection and were maintained in RPMI-1640 containing 10% FBS with 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured in a humidified 5% CO_2 incubator at 37˚C.

Real-time quantitative reverse transcription (RT)-PCR. Real-time quantitative RT-PCR was performed using a LightCycler® System and a LightCycler® 480 Probes Master kit (both from Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol with the following specific CHD8 primers: forward, 5'-AGTGGTGTCTACGTT TGGTGTG-3' and reverse, 5'-GATGGGCTCAATGAACAG GT-3'. CHD8 levels were normalized to GAPDH (primers: forward, 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3').

siRNA transfections and proliferation assays. For siRNA knockdown studies, double-stranded RNA duplexes targeting human CHD8 (5'-AGGAGCGUCCAGUAGAUGAACACG C-3'/5'-GCGUGUUCAUCUACUGGACGCUCCU-3'; 5'-UU CAAAUGCUUAAACUUUGGGAUUG-3'/5'-CAAUCCCAA AGUUUAAGCAUUUGAA-3') were purchased from Invitrogen (Carlsbad, CA, USA) (Stealth RNAi). Negative control siRNA (NC) was also purchased from Invitrogen. MKN-45 and NUGC4 cells were transfected with siRNA at a concentration of 20 μmol/l using Lipofectamine reagent (RNAiMax) in glucose-free Opti-MEM (both from Invitrogen). For proliferation assays, siRNA-, NC- or mock-transfected MKN-45 cells were seeded at $8.0x10^3$ cells/well in 96-well flat-bottomed microtiter plates in a final volume of 100 μl of culture medium per well. Cells were incubated in a humidified atmosphere (37˚C and 5% CO_2) for 24, 48, 72 or 96 h after initiation of transfection. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics Corp.) was then used to measure cell growth inhibition according to standard protocols. Briefly, after incubation, 10 μl of MTT labeling reagent (final concentration of 0.5 mg/ml) was added to each well, and the plate was incubated for an additional 4 h in a humidified atmosphere. Solubilization solution (100 μl) was added to each well, and the plate was incubated overnight in a humidified atmosphere. After confirming that the purple formazan crystals were completely solubilized, the absorbance of each well was measured by a Model 550 Series Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm corrected to 655 nm. Each sample was run with 6 replicates.

Gene set enrichment analysis (GSEA) of gastric cancer expression. To investigate CHD8 function in gastric cancer, we obtained gastric cancer expression profiles from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (accession code GSE22377) and analyzed these profiles using GSEA.

Expression profiles were normalized with the 'affy' R/BioConductor package (http://www.bioconductor.org/packages/release/bioc/html/affy.html). We applied a continuous-type CLS file with the CHD8 profile to phenotype labels in GSEA. The metric for ranking genes was set as 'Pearson' and all other parameters were set to their default values.

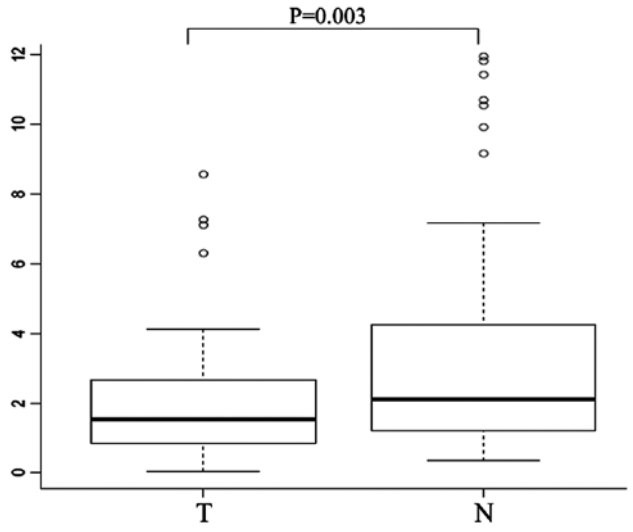

Figure 1. Analysis of chromodomain helicase DNA-binding 8 (*CHD8*) mRNA expression in gastric tumor tissues (T) and the corresponding normal mucosa (N) by real-time reverse transcription-PCR. *CHD8* mRNA was significantly downregulated in T compared with N (P=0.003).

Statistical analysis. The significance of differences between 2 groups was estimated with the Student's t-test and Chi-square test. Overall survival curves were plotted according to the Kaplan-Meier method, with the log-rank test applied for comparison. Variables with a P-value of <0.05 by univariate analysis were used in subsequent multivariate analysis on the basis of the Cox proportional hazards model. All differences were considered statistically significant at the level of P<0.05. Statistical analyses were conducted using JMP 5 Software (SAS Institute).

Results

CHD8 expression in gastric cancer tissues. We examined *CHD8* mRNA expression in tumor tissues and the corresponding normal mucosa collected from 101 gastric cancer patients by quantitative real-time PCR. Notably, *CHD8* was significantly downregulated in tumor tissues when compared to the level in the corresponding normal mucosa (median *CHD8/GAPDH* ratio, 1.58 vs. 2.26, respectively; P=0.003) (Fig. 1).

Association between CHD8 mRNA expression and clinicopathological factors. As shown in Table I, we divided the patient population into a high (n=60) and a low *CHD8* expression group (n=41), with a cutoff value of 1.29 for the *CHD8/GAPDH* ratio in the cancerous tissues. The low *CHD8* expression group was significantly associated with an increased

Table I. Association between *CHD8* mRNA expression and clinicopathologic factors.

Factors	Low expression (n=41)	High expression (n=60)	P-value
Age (mean ± SD)	63.9±11.3	67.2±10.6	
Gender			0.013
Male	31	31	
Female	10	29	
Histological grade			0.44
Well and mod	18	31	
Por and sig	23	29	
Depth of invasion			0.036
T1-2	12	30	
T3-4	29	30	
Lymph node metastasis			0.028
Negative	11	29	
Positive	30	31	
Lymphatic invasion			0.23
Negative	28	34	
Positive	13	26	
Venous invasion			0.71
Negative	8	10	
Positive	33	50	
Peritoneal dissemination			0.051
Negative	32	55	
Positive	9	5	

CHD8, chromodomain helicase DNA-binding 8; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell adenocarcinoma.

depth of tumor invasion (P=0.036) and lymph node metastasis (P=0.028). Relative to the high *CHD8* expression group, the low *CHD8* expression group showed an increased tendency to be associated with peritoneal dissemination (Table I). No significant differences were observed regarding histological type, lymphatic invasion, or venous invasion.

Association between CHD8 expression and prognosis. With regard to overall survival, patients with high *CHD8* expression had a significantly better prognosis than those with low *CHD8* expression (P=0.044) (Fig. 2). Univariate analysis revealed that the level of *CHD8* expression, depth of tumor invasion and presence of lymph node metastasis, lymphatic invasion, or venous invasion were significantly correlated with prognosis in gastric cancer patients (Table II). These factors identified by univariate analysis were then applied to multivariate analysis, and *CHD8* expression was found to be an independent prognostic indicator for overall survival in patients with gastric cancer (P=0.048; Table II).

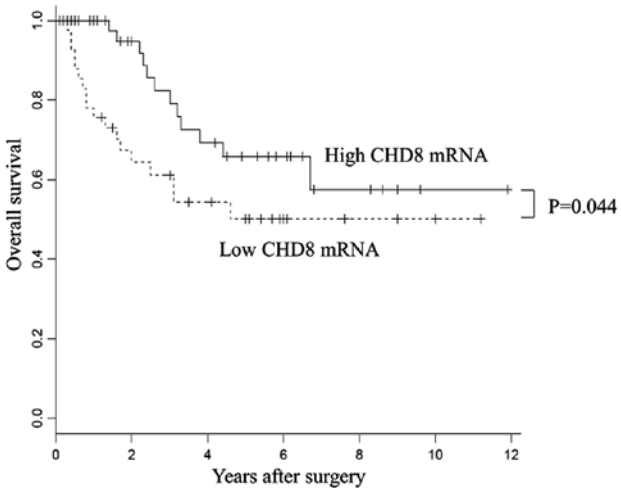

Figure 2. Kaplan-Meier survival curves for gastric cancer patients divided into subgroups according to the chromodomain helicase DNA-binding 8 (*CHD8*) mRNA expression. Patients in the low *CHD8* mRNA expression group had poorer survival than those in the high expression group (P=0.044).

GSEA for analyzing CHD8 function in gastric cancer. We used 2,996 gene sets, obtained from GSEA, Qiagen and Sabioscience websites (GSEA: http://www.broadinstitute.org/gsea/index.jsp; Qiagen: https://www.qiagen.com/geneglobe/pathways.aspx; Sabioscience: http://www.sabiosciences.com/pathwaycentral.php). The results of GSEA showed that 5 gene sets were significantly enriched, with a false discovery rate (FDR) of <10% (Table III). In gastric cancer tissues with low *CHD8* expression, genes in the Wnt/β-catenin pathway signature were the most highly enriched gene set (P=0.000, FDR=0.000) (Fig. 3). Similarly, genes within the cell cycle signature were also significantly enriched (P=0.041, FDR=0.069) (Fig. 3). Thus, *CHD8* expression was inversely correlated with the Wnt/β-catenin gene signature and the cell cycle gene signature (Fig. 4).

In contrast, p53-mediated apoptosis, which has been shown to be associated with CHD8 expression in embryonic cells (15), was not significantly associated with *CHD8* in gastric cancer (Figs. 3 and 4).

In vitro assessment of CHD8 knockdown. To investigate the role of *CHD8* in gastric cancer progression, *CHD8* expression was suppressed by transient siRNA transfection into MKN-45 and NUGC4 cells. The reduction in *CHD8* expression was confirmed by quantitative read-time RT-PCR. As revealed by MTT assays, siRNA-mediated knockdown of *CHD8* promoted the proliferation of MKN-45 and NUGC4 cells (MKN-45: P=0.028; NUGC4: P=0.016 vs. control cells) (Fig. 5).

Discussion

Our present study revealed that *CHD8* mRNA expression was frequently downregulated in gastric cancer tissues compared with that in the adjacent normal mucosa. In addition, we found that *CHD8* played a key role as a tumor suppressor in gastric cancer, i.e. high expression of *CHD8* was associated with a better prognosis. By *in vitro* analysis, we confirmed that knockdown of *CHD8* mRNA in gastric cancer cell lines resulted

Table II. Univariate and multivariate analyses for overall survival using the Cox proportional hazards regression model.

	Univariate analysis			Multivariate analysis		
Factor	RR	95% CI	P-value	RR	95% CI	P-value
Age (>65 years)	0.83	0.58-1.20	0.336	-	-	-
Gender	0.97	0.67-1.43	0.89	-	-	-
Histology grade (well and mod/por and sig)	1.13	0.79-1.66	0.48	-	-	-
Tumor stage (T1/T2-4)	2.39	1.49-4.41	0.00001	1.08	0.57-2.23	0.81
Lymph node metastasis (negative/positive)	3.46	1.89-8.63	0	2.17	1.04-5.74	0.037
Lymphatic invasion (negative/positive)	2.39	1.42-4.90	0.0004	1.74	0.94-3.78	0.076
Venous invasion (negative/positive)	2.11	1.43-3.04	0.0004	1.8	1.13-2.84	0.012
CHD8 mRNA expression (low/high)	1.44	1.00-2.10	0.047	1.55	1.00-2.45	0.048

RR, relative risk; CI, confidence interval; well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet ring cell adenocarcinoma; *CHD8*, chromodomain helicase DNA-binding 8.

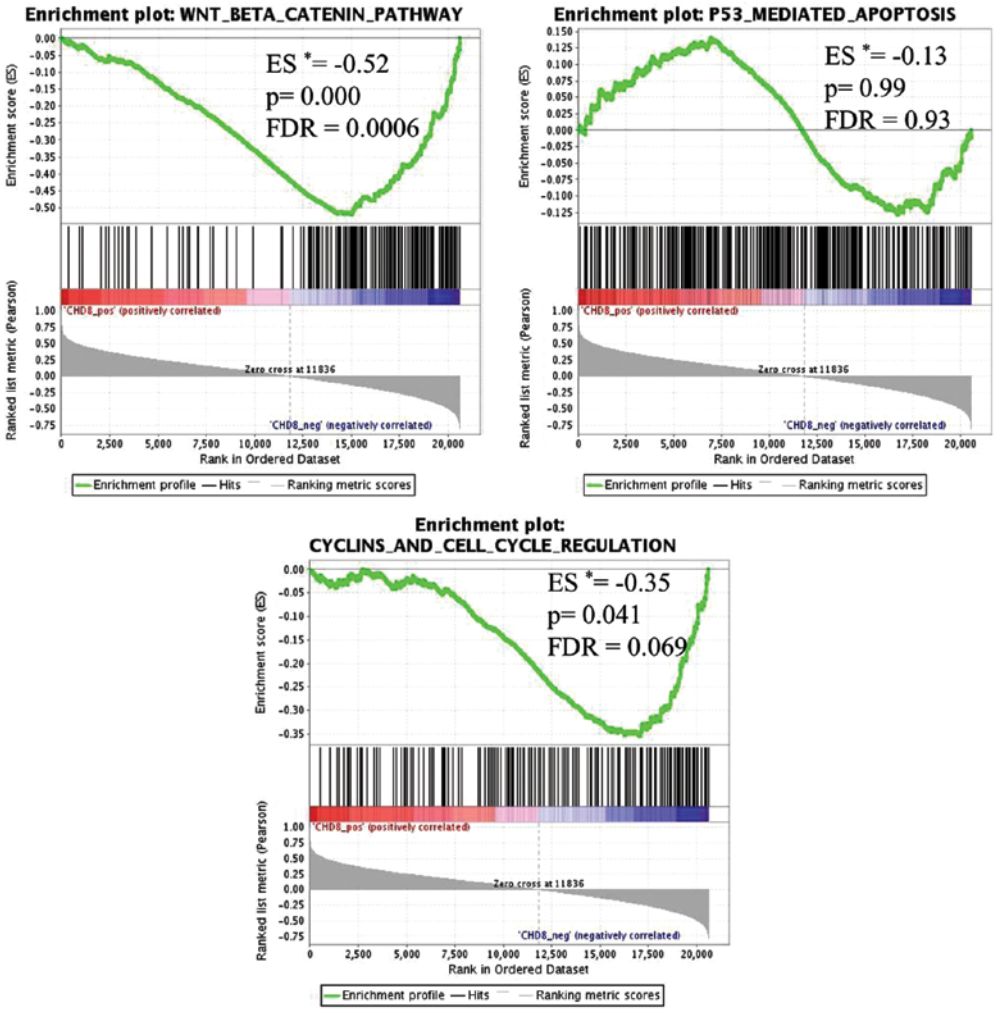

Figure 3. Enrichment plots of gene expression signatures for Wnt/β-catenin, cell cycle and p53-mediated apoptosis pathways according to chromodomain helicase DNA-binding 8 (*CHD8*) mRNA expression levels. The bar-code plot indicates the positions of genes in each gene set; red and blue colors represent positive and negative Pearson correlations with *CHD8* expression, respectively. ES, enrichment score; FDR, false discovery rate.

in more aggressive proliferation. Moreover, GSEA based on GEO expression array data showed that *CHD8* expression was significantly associated with downregulation of genes involved in the Wnt/β-catenin pathway and the cell cycle. Thus, loss of *CHD8* expression was expected to enhance activation of the Wnt/β-catenin pathway and promote cell cycle progression in

Table III. Result of GSEA for *CHD8* expression in gastric cancer.

Gene set	ES	Nominal P-value	FDR q-value
WNT_BETACATENIN_PATHWAY	-0.52	0.000	0.0006
GUTIERREZ_MULTIPLE_MYELOMA_DN	-0.71	0.000	0.050
ALONSO_METASTASIS_UP	-0.55	0.000	0.069
CYCLIN_AND_CELL_CYCLE_REGULATION	-0.35	0.041	0.069
CHIANG_LIVER_CANCER_SUBCLASS_UNANNOTATED_DN	-0.72	0.000	0.074

GSEA, gene set enrichment analysis; *CHD8*, chromodomain helicase DNA-binding 8; ES, enrichment score; FDR, false discovery rate.

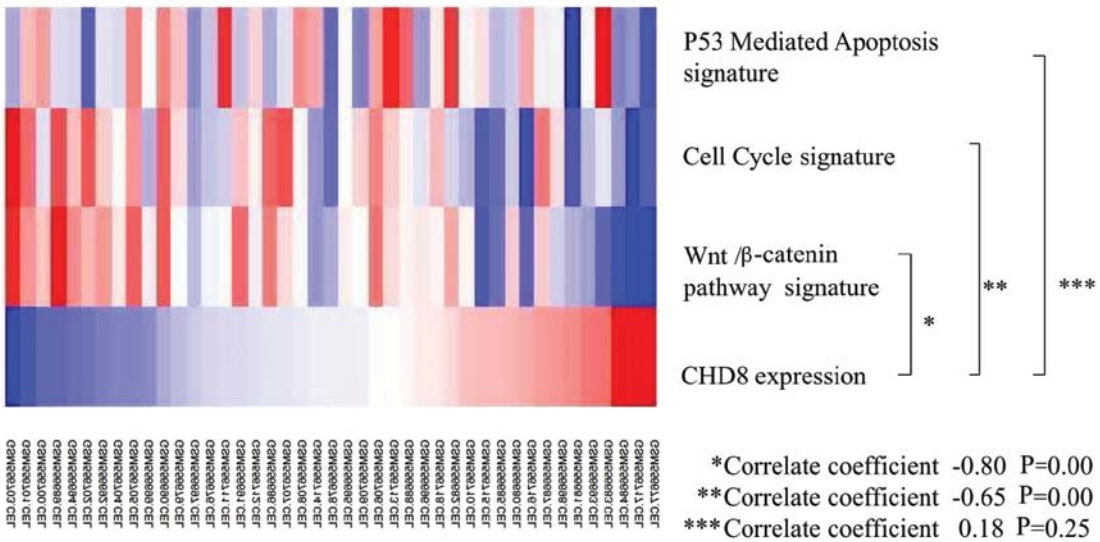

Figure 4. Heat map of gene expression averages for 44 gastric cancer samples enriched in the Wnt/β-catenin pathway gene expression signature. Enriched gene expression averages were classified by high and low chromodomain helicase DNA-binding 8 (*CHD8*) expression levels. The red and blue colors indicate high and low expression, respectively.

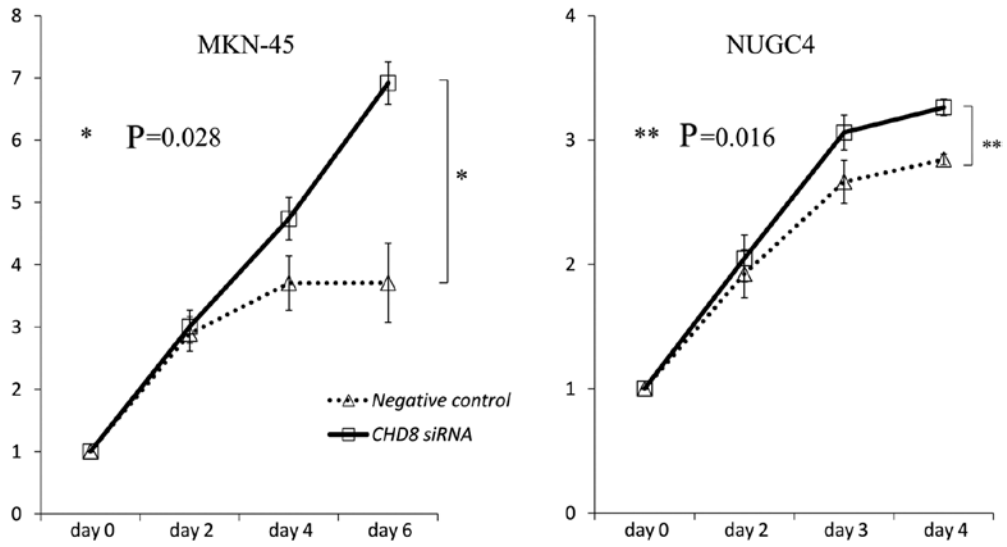

Figure 5. Knockdown of chromodomain helicase DNA-binding 8 (*CHD8*) promoted proliferation of MKN-45 and NUGC4 cells. Cell growth was measured on day 2 (48 h), day 3 (72 h) and day 4 (96 h) by MTT assay. Absorbance at day 0 was assigned a value of 1. The results are the means ± SD of 6 replicates.

gastric cancer. Taken together, these data suggest that CHD8 exerts a suppressive effect on cell proliferation through negative regulation of the Wnt/β-catenin pathway and cell cycle progression in gastric cancer.

From the GSEA study, we found significant associations between *CHD8* expression and Wnt/β-catenin signaling and the cell cycle; this result is consistent with several studies that have described CHD8 as a negative regulator of the Wnt/β-catenin pathway. For example, Thompson *et al* showed that *CHD8* is an ATP-dependent chromatin remodeling factor that regulates β-catenin target genes in colon cancer cells (HCT116) (8). Additionally, Nishiyama *et al* (16) suggested that CHD8 negatively regulates β-catenin function by recruiting histone H1 to the promoters of Wnt target genes. Together with our current data, these studies suggest that CHD8 is an essential mediator of Wnt/β-catenin signaling in several different cellular contexts.

Studies have also suggested that CHD8 controls the expression of cyclin E2 (*CCNE2*) and thymidylate synthetase (*TYMS*), two genes expressed during G_1/S transition of the cell cycle (11). Indeed, in the present study, both genes were upregulated in gastric cancer tissues with low *CHD8* expression (data not shown). Moreover, a previous study also found that p53-dependent apoptosis was suppressed by histone H1, which is recruited by CHD8 during embryogenesis (15); however, to date, no studies have investigated the involvement of CHD8 in p53 activation and signaling in malignancies. Our study demonstrated that *CHD8* expression was not associated with the p53 pathway in gastric cancer.

The Wnt/β-catenin signaling pathway is an important functional pathway in development, specification of cell fate and adult stem cell proliferation (17-20). Abnormal Wnt signaling has been demonstrated in a variety of human cancers. For example, Ooi *et al* (21) demonstrated that 3 oncogenic pathways (proliferation/stem cell, NF-κB and Wnt/β-catenin) were deregulated in the majority of gastric cancers and that increased activation of the Wnt/β-catenin pathway was associated with poor patient survival in gastric cancer. The present study is the first report to show that CHD8 interacts with the Wnt/β-catenin pathway in gastric cancer, with low *CHD8* mRNA expression contributing to a poor prognosis.

In conclusion, our data demonstrate that CHD8 functions as a tumor suppressor by regulating Wnt/β-catenin signaling and the cell cycle. Moreover, loss of *CHD8* expression, commonly observed in gastric cancer, may represent a novel indicator for the biological aggressiveness of gastric cancer.

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