Human ZNF312b Promotes the Progression of Gastric Cancer by Transcriptional Activation of the K-ras Gene

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Abstract

Gastric cancer ranks second among the most common causes of cancer deaths worldwide. Recent studies reported target molecules that are candidates for new therapeutic interventions; however, their molecular mechanism has not been clearly defined. In this study, we found that ZNF312b plays a role in tumor progression and metastasis in gastric cancer via transcriptional activation of the K-ras oncogene. ZNF312b seems to be specifically overexpressed in gastric cancer tissues and cell lines. The overexpression of ZNF312b induces cancer-like phenotypes, including accelerated proliferation and increased tumor masses in nude mice, which are completely reversed by its knockdown in gastric cancer cell lines, implying direct involvement in gastric tumor progression. From analyses using deletion mutants of ZNF312b and K-ras promoter-driven luciferase reporters, we found that it translocates into the nucleus via the proline-rich domain of its COOH terminus to activate transcription of the K-ras gene, resulting in an enhancement of the extracellular signal-regulated kinase signaling pathway that governs cell proliferation. Taken together, these results suggest that ZNF312b contributes to the promotion of gastric cancer by triggering K-ras oncogene expression. The current study is the first to report that ZNF312b, a novel transcription factor, was associated with tumorigenicity of gastric cancer. This might be a valuable target that could provide new insight into the development of new therapeutic modalities for patients with gastric cancer. [Cancer Res 2009;69(7):3131–9]

Introduction

Gastric cancer ranks second among the most common causes of cancer deaths worldwide. In recent years, knowledge about the molecular features of gastric carcinoma has increased rapidly. Loss of heterozygosity at loci on chromosomes 1, 5, 7, 12, and 17 has frequently been identified in advanced gastric carcinomas (1). Genetic alteration, such as activation of oncogenes and inactivation of tumor suppressor genes, has often been found in gastric cancer. Mutation or amplification of the oncogenes K-ras, c-erbB-2, K-sam, c-met, and c-myc and inactivation of the tumor suppressor genes p53 and adenomatosis polyposis coli are believed to be related to the development of gastric cancer. These genes are potential prognostic markers used to predict the progression and metastasis of gastric cancer (1–3). In addition, a recent technique using the comprehensive analysis of gene expression patterns has distinguished tumor subgroups and identified genes whose expression may be correlated with disease classification, early detection, prognostication, as well as progression of gastric cancer (4–6).

We identified a large number of genes expressed in human gastric cell lines or tissues, which had been identified as candidate genes related to human gastric cancer (7, 8), to elucidate the molecular mechanisms of human gastric cancer. Recently, as a gain-of-function approach, we have done large-scale functional screening using a zebrafish system in which in vitro synthetic mRNAs for target genes were microinjected into zebrafish embryos.3 During this process, we identified a human ZNF312b gene that was associated with phenotypic changes, such as headlessness in zebrafish, and was highly expressed in gastric cancer cells.

Human ZNF312b is a transcription factor that encodes 475 amino acids and contains six C2-H2–type zinc finger domains. ZNF312b was originally isolated as an anterior neuroectoderm-specific gene in both Xenopus and zebrafish (9, 10). The ZNF312b is named Fezf1 because it highly identifies with forebrain embryonic zinc finger protein 1 (Fezf1) of the Xenopus (10). The Fezf family includes Fezf1 and Fezf2, Fezf2 is assigned as ZNF312. Human ZNF312b has an identity of 55% for human ZNF312. In mice and zebrafish, ZNF312b and ZNF312 are expressed in overlapping domains in the forebrain during development (9–12). ZNF312b has only been known to non–cell-autonomously regulate development of the olfactory bulb and to serve as a novel marker with spatially distinct patternings in the neonatal ventromedial hypothalamus (11, 13).

In this study, we have shown that human ZNF312b is an essential component of gastric tumor development because it promotes extracellular signal-regulated kinase (ERK) activation by mediating the overexpression of oncogenic K-ras. These data reveal a novel role for ZNF312b as a transcription factor for oncogenic genes, such as K-ras, in human gastric cancer.

Materials and Methods

Reagents. Antibodies to ERK1/2, phospho-ERK1/2 (E10), mitogen-activated protein/ERK kinase 1/2 (MEK1/2; L38C12), and phospho-MEK1/2 (41G9) were purchased from Cell Signaling Technology, antibodies to ERK2 (C-14) and K-ras (F234) were purchased from Santa Cruz Biotechnology; antibody to hemagglutinin (HA) epitope was purchased from Roche; mouse monoclonal antitubulin antibody and myelin-binding

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protein were purchased from Sigma; anti-lamin B antibody was obtained from Calbiochem; horseradish peroxidase–conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Pierce.

**Patient tissue samples.** The human cancer tissues and adjacent nontumorous tissues from stomach (45 pairs), liver (24 pairs; stage I: 6 pairs, stage II: 6 pairs, stage III: 6 pairs, stage IV: 6 pairs), colon (40 pairs, stage II), and lung (10 pairs, stage III) were obtained for gene expression examination from the College of Medicine, Chungnam National University, with informed consent. The tumors were staged according to the tumor-node-metastasis classification of the Union Internationale Contre le Cancer.

**Cell culture.** Human gastric cancer cell lines SNU1, SNU16, SNU216, SNU484, SNU601, SNU638, SNU668, and SNU719 were originally obtained from the Korean Cell Line Bank (14, 15), and these cells were routinely cultured at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 with 10% fetal bovine serum (Sigma).

**Construction of stable cell lines.** To generate stably transfected cell lines, SNU638 and SNU668 cells (1 × 10^6 in 60-mm dishes) were transfected with 4 μg of either pcDNA3.1 or pcDNA3.1-HA-ZNF312b using FuGene 6 (Roche), and to generate stably ZNF312b knocked down cell lines, SNU638 and SNU668 cells (1 × 10^6 in 60-mm dishes) were transfected with 4 μg of either pSilencer (Ambion) or pSilent-shZNF312b using FuGene 6. Transfected cells were selected in medium containing G418 (0.3 mg/mL, SNU638; 0.6 mg/mL, SNU668). The selected clones were maintained in medium containing G418 (0.3 mg/mL).

**Proliferation assay.** 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2H-tetrazolium salt (WST-8; CCK-8, Dojindo) assay was done to evaluate the rate of cell proliferation, as described previously (16). Briefly, log-phase cells were trypsinized into a single-cell suspension and plated into 96-well plates at a density of 1 × 10^3 per well. CCK-8 solution was added to each well. After 1 h, the absorbance at 450 nm of each well was read on a microplate reader (Perkin-Elmer).

**Cell cycle analysis.** Cells in log phase were washed twice with PBS, fixed with 70% ethanol, and incubated overnight at 4°C. They were then resuspended at 37°C for 30 min in propidium iodide staining solution [PBS containing 40 μg/mL DNase-free RNase A (Roche) and 40 μg/mL propidium iodide (Invitrogen)] and prepared fresh daily. The cells were analyzed for fluorescence intensity by FACSscan (BD Biosciences) using CellQuest flow cytometric analysis software. The cell proliferation index (PI) was calculated using the following formula: 

\[ PI = \frac{\text{number of S-, G2-, and M-stage cells}}{\text{number of total cells}} \times 100\% \]

Each study was repeated four times.

**Real-time reverse transcription-PCR.** The total RNA of tumor, nontumor tissues, and cell lines was extracted using Trizol reagent (Invitrogen). cDNA (1 μg) was reverse transcribed using SuperScript II (Invitrogen). The PCR was done using the first cDNAs as templates and specific primers for the target genes (Supplementary Table S1). The amplification was carried out using Exicycler version 2 (Bioneer Co.) in a 10 μL reaction mixture containing 4 μL of diluted DNA template, 2 pmol of each primer, and 5 μL of 2× SYBR Premix Ex Taq (TaKaRa).
Biotechnology). The β-2-microglobulin (B2M) gene was used as the control, and the reaction of each sample was done in triplicate. Using the comparative threshold cycle (Ct) method (17), the relative quantification of gene expression was calculated as the ratio of cancer tissue/noncancer tissue after normalization against B2M for each sample. Each PCR product for target genes was confirmed as a single band with an expected size on 1% agarose gel.

Immunocytochemistry. SNU668 cells were plated onto glass coverslips and transfected with plasmids of HA-ZNF312b or deletion constructs. After 24 h, the cells were fixed in 4% paraformaldehyde for 20 min. The cells were then treated with 1% Triton X-100 for 10 min and 1% bovine serum albumin for 1 h at room temperature before incubation overnight with a monoclonal anti-HA at 4°C. The cells were incubated with an Alexa Fluor 568–conjugated antiserum secondary antibody for 1 h, washed thrice with PBS, and incubated with 4,6-diamidino-2-phenylindole (DAPI) for 30 min. The cells were mounted onto slides and analyzed using an Olympus fluorescent microscope.

Luciferase assay. Luciferase assays were done with the Promega Luciferase Assay System according to the manufacturer’s specifications. The cells were cotransfected with the K-ras promoter luciferase reporter and control β-galactosidase (β-gal) plasmids for 48 h and harvested. Using an enhanced luciferase assay kit (Promega), luciferase activity was measured and then the relative luciferase activity was calculated by normalizing luciferase activity to the β-gal activity. To generate luciferase reporter constructs, K-ras promoters of various sizes were PCR amplified from normal human genomic DNA (Clontech) using primers designed to contain KpnI or Bgl II linker sequence (Ras2.0: forward, 5'-ggggtacgcgatcagacagccccgg-3'; Ras0.5: forward, 5'-ggggtacccaacacagactccgggta-3'; Ras0.65: forward, 5'-ggggtacgagacctgcagatggctg-3'; Ras0.5: forward, 5'-ggggtacctccttcttcgccgacg-3') and reverse, 5'-cgagatctgctgccggcagtaagccgac-3'). The PCR products were cloned into the KpnI and BgII sites of the pG3-basic vector (Promega).

Soft agar clonogenic assay. Anchorage-independent growth was assessed by soft agar clonogenic assay as described previously (18). Briefly, cells were detached and plated in 0.3% agarose with a 0.6% agarose underlay (1 × 10³ per well). The number of foci >100 μm in 10 randomly selected fields was counted under the microscope after 15 d.

Mouse experiments. Mice were handled using the best humane practices and were cared for in accordance with NIH Animal Care and Use Committee guidelines. Cells were harvested from 1000 culture dishes, using trypsin, and resuspended in PBS. Mice were injected sc with 5 × 10⁶ cells in 0.1 mL into the right upper back and raised for the following 30 d. The mice were then monitored for tumor volume and overall health. The size of the tumor was determined by caliper measurement of the s.c. tumor mass. Tumor volume was calculated according to the formula 0.5 × length × width². Each experimental group contained four mice. Two independent experiments were done and gave similar results.

DNA-binding assay of ZNF312b. The DNA-binding activity of ZNF312b was assessed using Trans-AM Flexi transcription factor assay kits (Active Motif; ref. 19) according to the supplied protocol. The biotinylated oligonucleotide 5'-biotin-ttgttatattatctagatgatagatgatgatgcggcagtaagccgac-3', 1 pmol per reaction, was used.

Statistics. Data were analyzed using a Student’s t test on SigmaPlot 8.0 software; the P value was derived to assess the statistical significance. Data of all figures represent the means ± SD of three independent experiments.

Results

ZNF312b is overexpressed in human gastric cancer cells. The data from real-time reverse transcription-PCR (RT-PCR) using 45 pairs of human cancer samples and matched surrounding noncancerous tissues revealed that ZNF312b was overexpressed in most of the gastric cancer tissues compared with the

Figure 2. ZNF312b has tumorigenic ability. A and B, the stable cell lines [ZNF312b-reduced (A) and HA-ZNF312b–overexpressed (B) cells] were placed in medium containing soft agar and incubated for 20 d. The number of low-power fields (>100) in the microscope was counted in 10 random fields. C, tumorigenic ability was evaluated by tumor growth using athymic nude mice. Mice were injected sc with 5 × 10⁶ stable transfected SNU668-Neo or SNU668-ZNF312b cells. After 30 d of cell injection, the volume of the tumor was calculated according to the formula 0.5 × length × width². Representative data (n = 3) are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
necancerous tissues. A 2- to 20-fold increase of ZNF312b, compared with that of noncancerous tissues, was observed in >50% of cancer tissues (Fig. 1A). In accordance with the results, ZNF312b was also highly expressed in all of the gastric cancer cell lines compared with those of the Hs. 677st cell line, a normal gastric cell line (Supplementary Fig. S1A). In contrast, ZNF312b was not observed or was expressed at very low levels in other cancer tissues from human liver, colon, and lung patients compared with that of matched noncancerous tissues (Supplementary Fig. S1B). In addition, its no or low expression level was confirmed in other human tissues, except for normal brain tissues by Northern blot (data not shown). These results indicated that high expression of ZNF312b may be specific for gastric cancer cells.

ZNF312b regulates proliferation of human gastric cells. To assess the role of ZNF312b in gastric tumors, we prepared three small interfering RNAs (siRNA) targeted to the coding region of ZNF312b and examined their knockdown effect in cell proliferation. As shown in Supplementary Fig. S2A and B, siZNF312b-1 was shown to effectively knock down ZNF312b and inhibit proliferation of SNU638 cells compared with siZNF312b-2 and siZNF312b-3. When SNU638 cells were transfected with siZNF312b-1, it induced a marked decline in cell proliferation of SNU638 cells (Fig. 1B, left). The cell cycle analysis also revealed that the population of cells in the S + G2 + M phase indicating the proliferation rate was decreased from 61% to 49% by knocked down ZNF312b (Fig. 1B, right). In the siZNF312b-1–transfected cells, cell death, such as necrosis and apoptosis, was not observed (data not shown). In addition, we constructed an SNU668 stable cell line transfected with shZNF312b, which are short hairpin RNA vector based on the siZNF312b-1 sequence. The results showed that SNU668-shZNF312b cells grew significantly more slowly than the control cells, SNU668-Silent (Supplementary Fig. S2C and D). These results show that ZNF312b is an essential regulator of cell proliferation in gastric cancer cells.

To further investigate the relationship of ZNF312b in cell proliferation, we constructed stably HA-tagged ZNF312b-express-
ing gastric cancer cells using SNU638 and SNU668 cell lines. The results, shown in Fig. 1C, revealed that overexpression of ZNF312b significantly increased cell proliferation, especially in SNU668-ZNF312b cells. In addition, analysis of their cell cycle showed that ZNF312b-overexpressing cell lines markedly increased their population in the S + G$_2$ + M phase of the cell cycle (Fig. 1D). These results indicate that ZNF312b regulates proliferation of human gastric cancer cells.

**ZNF312b acts as a regulator of tumorigenicity in human gastric cancer cells.** To examine the ability of ZNF312b to cause a modulation of human gastric cancer cell growth in soft agar, an anchorage-independent growth assay was done. As shown in Fig. 2A, SNU668-shZNF312b cells resulted in a significant reduction in the colony number compared with SNU668-Silent. On the other hand, ectopic expression of ZNF312b increased the number of colonies >2-fold (in SNU638 cells) and 5-fold (in SNU668 cells) in comparison with those of control cells (Fig. 2B). To confirm these effects in vivo, we s.c. injected HA-ZNF312b–expressing SNU668-ZNF312b cells into the right upper back of athymic nude mice. On experiment day 30, the average tumor volume in control animals was 1,226 mm$^3$ compared with 18,236 mm$^3$ in the SNU668-ZNF312b group (Fig. 2C). Collectively, these results suggest that ZNF312b regulates cell proliferation and tumor growth.

**ZNF312b is translocated into the nucleus via the COOH terminus and promotes the transcriptional activation of the K-ras gene.** Because zinc finger protein has been known to be a transcriptional activator, we investigated target genes activated by ZNF312b to elucidate its physiologic role. In particular, transcription level of target genes related to cell proliferation were examined because of the increase in cell proliferation by overexpression of ZNF312b. Interestingly, Fig. 3A shows that the oncogenic K-ras gene was dramatically overexpressed in SNU668-ZNF312b cells. It was

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**ZNF312b Activates K-ras Gene**

**Figure 4.** ZNF312b activates K-ras transcription by interaction of the ZNF-binding region of the human K-ras promoter with its COOH terminus. A, the various deletion fragments of the K-ras promoter were cloned in frame into the pGL3. The ZNF312b probe indicates an oligomer containing the expected specific binding sequence of ZNF312b in the K-ras promoter. B, SNU638 cells were cotransfected with the pGL-Ras2.0 and deletion fragments of ZNF312b. The cells were harvested and then used for the luciferase and β-gal assays. The luciferase activity was calculated as described in Materials and Methods. C, SNU638 cells were cotransfected with various K-ras reporter plasmids and β-gal plasmid along with either mock or C1 fragment. The luciferase activities were measured and calculated as described in B. D, the DNA-binding activity of ZNF312 against the K-ras promoter was measured using the transcription factor binding assay kit, as described in Materials and Methods. Expression levels of proteins are shown in the HA blot. **, $P < 0.01$; ***, $P < 0.001$. 

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also revealed that target genes of the Ras/ERK signaling pathway, such as MMP-1, TIMP-1, and CDK2, were highly overexpressed, whereas the p27kip1 gene was reduced. Surprisingly, although the K-ras gene was not the target gene of the ERK pathway activated by epidermal growth factor (EGF) stimulation, the K-ras gene was dramatically overexpressed by ZNF312b expression. These data propose that ZNF312b might be a transcriptional factor for the K-ras gene, and activation of the Ras/ERK signaling pathway might be related to an increase in the proliferation of gastric cancer cells by ZNF312b.

To confirm the function of ZNF312b as a transcriptional activator, analyses of domain and localization for ZNF312b were done. The 52-kDa ZNF312b protein contains six C2-H2-type zinc finger domains and a proline-rich repeat sequence in the COOH-terminus. Based on this information, a series of fragments of ZNF312b with only the NH2 terminus or COOH terminus were fused in frame to the HA-tag in the pcDNA3-HA plasmid, and four deletion constructs were made (Fig. 3B). The localization analysis of these constructs in SNU638 cells revealed that the ZNF312b containing the NH2 terminus was localized only in the cytosolic fraction, whereas ZNF312b containing the COOH terminus translocated into the nucleus (Supplementary Fig. S3). In particular, the COOH terminus (amino acids 341–475) containing the proline-rich repeat sequence played a key role in translocation into the nucleus. The results were also confirmed in immunocytochemistry data (Fig. 3C).

To determine whether the ZNF312b protein can function as an activator of the cis-element of K-ras, a 2-kb promoter region and series of deletion fragments were inserted into the pGL3 (Fig. 4A). As expected, cotransfection of the reporter plasmid Ras2.0, together with constructs of FL, C1, and C2, resulted in the induction of luciferase activity. In contrast, the constructs of N1 and N2 abolished the activator activity of ZNF312b (Fig. 4B). In addition, Fig. 4C shows that the Ras0.5 construct reduced the relative luciferase activity, whereas other K-ras constructs did not. This result indicates that ZNF312b was bound in the −650-bp to −500-bp region of the K-ras promoter for K-ras transcription. We also examined direct DNA-binding activity of ZNF312b in the region. Figure 4D shows that DNA-binding activity in overexpressed cells of ZNF312b containing FL or C1 was much higher than that in the cells that expressed a mock or N1. These data indicate that ZNF312b is a hitherto unknown transcription factor in gastric cells.

ZNF312b activates the ERK signaling pathway. To examine how ZNF312b regulates activation of the ERK signaling pathway, we investigated the effect of siRNA of ZNF312b on the EGF-mediated ERK activation pathway. Western blot analyses of Fig. 5A (top) showed that ERK was activated by EGF stimulation, and ERK phosphorylation was dramatically retarded in siZNF312b–transfected cells, whereas it exhibited typical kinetics in the control cells. Consistent with this result, EGF stimulation of ZNF312b stably knocked down SNU668-shZNF312b cells, which resulted in a decrease in the level of phosphorylated ERK (Fig. 5A, bottom). On the other hand, ZNF312b overexpressed cells were shown to enhance phosphorylation of ERK (Fig. 5B). Interestingly, these data also indicate that ZNF312b is able to induce ERK activation without stimuli. That is, the ERK phosphorylation was retarded in the ZNF312b-decreased cells and dramatically enhanced in the ZNF312b-increased cells (0 minute in Fig. 5A and B). These results suggest that ZNF312b expression can regulate ERK activation in a dose-dependent manner, either alone or in synergy with EGF treatment. Taken together, these data indicate that ZNF312b activates the Ras/ERK signaling pathway.

ZNF312b activates the K-ras/ERK signaling pathway by the induction of K-ras. Finally, the induction of the endogenous K-ras gene was examined in ZNF312b-transfected cells. The induction of endogenous K-ras by ZNF312b was dramatically increased in SNU638 cells that overexpressed ZNF312b containing the COOH-terminal region (Fig. 6A, left). Moreover, ERK1/2 activation was dramatically induced in these cells (FL, C1, and C2). The phosphorylation of MEK1/2 was also slightly increased. We also confirmed that K-ras expression and ERK activation were reduced by knock down of ZNF312b (Fig. 6A, right). We also examined whether ZNF312b has tumorigenic functions ex vivo. As shown in Fig. 6B, cell proliferation was increased by the transiently or stably ectopic expression of ZNF312b containing a COOH-terminal region. Consistently, when the soft agar clonogenic assay was done, the colony number was increased >2-fold in comparison with those of SNU638-Neo and SNU638-N1 cells (Fig. 6C). These results clearly show that the activation of the Ras/ERK pathway via overexpressed
ZNF312b plays a significant role in cell proliferation and tumorigenicity as biological processes.

Taken together, these data support our hypothesis that ZNF312b induces K-ras expression by binding in the ZNF-binding region of a human K-ras promoter and then activates ERK via MEK1/2, although phosphorylation of MEK1/2 was slightly increased (Fig. 6D).

Discussion

This study is the first report showing the relationship of human ZNF312b and carcinogenesis. In this study, we found that human ZNF312b is a transcription factor that activates transcription of the K-ras gene. The transcriptional activation was triggered by binding of the ZNF domain in the COOH-terminal region of ZNF312b into the ZNF-binding site (−650 to −500) of the K-ras gene promoter in the nuclei of gastric cancer cells. In addition, it was observed to be specifically overexpressed in gastric cancer cell lines and tissues, whereas it was not observed or was expressed at a very low level in most adult normal tissues as well as human cancers, except for primitive neuroectodermal tumors (Supplementary Fig. S1B). These data indicate that ZNF312b is a transcription factor that has specific oncogenic ability in gastric cancer cells.

The expression level of ZNF312b was observed to differ according to the gastric cancer cell lines. Consequently, we checked the doubling time of SNU638, a highly overexpressed cell line of ZNF312b, and SNU668, a low-expressed cell line of ZNF312b. The doubling time of SNU638 cells was found to be faster than that of SNU668 cells (data not shown). Moreover, the results of a cell proliferation and soft agar clonogenic assays of SNU668-ZNF312b cells were found to be dramatically increased compared with those of the SNU638-ZNF312b cells. In addition, it was shown that ERK phosphorylation in SNU668-ZNF312b cells was higher than that in SNU638-ZNF312b cells. These data provide strong evidence that ZNF312b activates the proliferation of gastric cancer cells. These findings were in accordance with our real-time PCR data on genes.

Figure 6. ZNF312b plays a significant oncogenic role by activation of the K-ras/ERK signaling pathway via induced K-ras in gastric cancer cells. A, left, SNU638 cells were transfected with deletion fragments of ZNF312b. The cells were lysed and subjected to immunoblot analysis with the antibodies to phospho-MEK1/2, MEK1/2, phospho-ERK1/2, ERK1/2, K-ras, and HA, respectively; right, SNU638 cells were transfected with 200 nmol/L of siZNF312b-1 or scrambled siRNA (si-Cont). The transfected cells were stimulated with EGF (20 ng/mL) for 10 min, and then cell lysates were subjected to immunoblot analysis as described above. B, left, the proliferation of SNU638 cells transfected with deletion fragments of ZNF312b was measured by CCK-8 assay at 48 h after transfection; right, the proliferation of SNU638 stable cells with deletion fragments of ZNF312b was measured by CCK-8 assay at the indicated time. C, the SNU638 stable cells with deletion fragments of ZNF312b and FL were done as described in Fig. 2. D, schematic representation of the tumorigenicity mechanism of ZNF312b. In gastric cancer, ZNF312b is abruptly overexpressed by unknown signals and then translocates into the nucleus via its COOH terminus. The COOH-terminal region of ZNF312b binds in the region of −650 to −500 of the K-ras gene promoter and then activates transcription of K-ras. The overexpressed K-ras activates its downstream signals, such as MEK and ERK, which result in the expression of target genes involved in tumor cell proliferation. **, \( P < 0.01; *** , P < 0.001. \)

induced by ZNF312b, in which CDK2, cyclin D1, and Cdc25a, activator genes of the G0–G1 phase, were highly overexpressed, whereas the p27kip gene, an inhibitor gene of the same phase, was repressed.

The ZNF motifs are widespread in the genome, and such motifs were present in multiple proto-oncogenes and in tumor suppressors (22). ZNF312b is a transcription factor containing six C2-H2-type zinc finger domains. The results of real-time PCR for cell proliferation–related genes showed that the K-ras gene was dramatically overexpressed. K-ras is well known as a key regulator of cell proliferation and differentiation. The ectopic expression using deletion constructs of ZNF312b and reporter assay using deletion constructs of the K-ras promoter revealed that the COOH-terminal region of ZNF312b mediated its translocation into the nucleus and induced K-ras–derived luciferase reporter expression. In addition, the binding site of ZNF312b was found to be located in −650 to −500 bp in the K-ras promoter. The site was also estimated to be a ZNF-binding domain by bioinformatic analysis. Collectively, our study results suggest that the COOH-terminal of ZNF312b is essential for its translocation into the nucleus, as well as for the induction of K-ras. It also indicates that ZNF312b plays a role as a novel transcription factor of the K-ras gene in gastric cells.

Ras was first identified as an oncogene and this ability has been well documented in many types of cells (23, 24). Mutations of Ras genes are present in ~30% of all human cancers, and K-ras mutations occur most frequently (25–27). Ras mutations lead to constitutive activation of Ras, and frequency and site of the mutation is known to differ according to human tumor type. In addition, a recent study reported that the genetic mechanism of carcinogenesis differs between the differentiated type and the undifferentiated type of gastric cancer and that K-ras mutations may be involved in the early stages of gastric carcinogenesis of the differentiated type (28). Based on these reports, we have done a study of K-ras mutation in human gastric cancer and its relationship to the expression level of ZNF312b. These studies may provide new insights into the control of the proliferation and growth of cancer cells based on Ras.

The Ras/Raf/MEK/ERK signaling cascade is one of the key signaling pathways that integrate extracellular stimuli into key biological responses controlling cell proliferation, differentiation, or death. This pathway is reportedly activated in >50% of acute myelogenous leukemia (29, 30) and acute lymphocytic leukemia and is also frequently activated in other cancer types, such as breast and prostate cancers. Our data show that in SNU668 cells, the phosphorylation of ERK and MEK1/2 was confirmed by overexpression of wild-type ZNF312b as well as its COOH-terminal mutant (C1 and C2). The parallel results were obtained by inhibition of ZNF312b using siRNA. In agreement with the above results, we also observed an increase in cell proliferation as well as in tumorigenicity by ectopic expression of ZNF312b-containing COOH terminus. According to our observations, the core region for cell proliferation is the COOH-terminal region containing the C2-H2 zinc finger domain. In addition, the prolifere-rich site in the COOH-terminal region is the essential domain for its translocation.

Our findings showed that ZNF312b is able to regulate the Ras/ERK pathway, thereby controlling proliferation of human gastric cells.

In preliminary experiments of ZNF312b, we examined the relationship of expression regulation of ZNF312b with epigenetic alternation using the histone deacetylase inhibitors trichostatin A (31) and sodium butyrate and the DNA methylation inhibitor 5-aza-2’-deoxycytidine (32). The result of real-time RT-PCR from the experiments revealed that ZNF312b expression was increased in gastric cancer cells treated with a DNA methylation inhibitor or with histone deacetylase inhibitors (Supplementary Fig. S4). In addition, the migration test showed that SNU668-ZNF312b cells increased the number of migrated cells, whereas ZNF312b knocked down SNU668-ZNF312b cells were reduced to the basal level of SNU668-Neo cells (Supplementary Fig. S5). These results indicate that ZNF312b was regulated by an epigenetic mechanism and its activation promotes aggressiveness and metastasis of cancer cells. To understand about these, we are doing studies about the metastasis and epigenetic mechanisms for ZNF312b.

In summary, ZNF312b induces transcription via binding of the zinc finger domain into the K-ras promoter and then promotes tumor growth by elevating ERK activation in gastric cancer cells. Our research offers important information relevant to therapies that target transcription and proliferation in gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

ZNF312b Activates K-ras Gene

Correction: Article on ZNF312b Activates K-ras Gene

In the article on how ZNF312b activates K-ras gene in the April 1, 2009 issue of Cancer Research (1), Dr. Cheol-Hee Kim should have been listed as co-corresponding author. Dr. Kim’s contact information is as follows: Department of Biology, Chungnam National University, Daejeon 305-764, South Korea. Phone: 82-42-821-5494; Fax: 82-42-822-9690; E-mail: zebrakim@cnu.ac.kr.


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