KIAA1324 Suppresses Gastric Cancer Progression by Inhibiting the Oncoprotein GRP78

Jin Muk Kang1, Sujin Park1, Staci Jakyong Kim1,2,3, Hyojung Kim1, Bona Lee1,4, Junil Kim1, Jinah Park1,5, Shin Tae Kim1,6, Han-Kwang Yang7,8, Woo Ho Kim7,9, and Seong-Jin Kim1,10

Abstract

Recent advances in genome and transcriptome analysis have contributed to the identification of many potential cancer-related genes. Furthermore, biological and clinical investigations of the candidate genes provide us with a better understanding of carcinogenesis and development of cancer treatment. Here, we report a novel role of KIAA1324 as a tumor suppressor in gastric cancer. We observed that KIAA1324 was downregulated in most gastric cancers from transcriptome sequencing data and found that histone deacetylase was associated with poor prognosis in gastric cancer patients. In the xenograft model, KIAA1324 significantly reduced tumor formation of gastric cancer cells and decreased development of preformed tumors. KIAA1324 also suppressed proliferation, invasion, and drug resistance and induced apoptosis in gastric cancer cells. Through protein interaction analysis, we identified GRP78 (glucose-regulated protein 78 kDa) as a KIAA1324-binding partner. KIAA1324 blocked oncogenic activities of GRP78 by inhibiting GRP78-caspase-7 interaction and suppressing GRP78-mediated AKT activation, thereby inducing apoptosis. In conclusion, our study reveals a tumor suppressive role of KIAA1324 via inhibition of GRP78 oncoprotein activities and provides new insight into the diagnosis and treatment of gastric cancer. Cancer Res; 75(15); 1-11. ©2015 AACR.

Introduction

Gastric cancer is the fourth most common type of cancer and the second leading cause of death from cancer worldwide (1). Although clinical treatment for gastric cancer has improved, gastric cancer therapy still remains challenging due to the difficulty in early detection of gastric cancer and complexities of the disease (2). Therefore, it is crucial to investigate novel genes that govern the development and progression of gastric cancer to elucidate the process of gastric carcinogenesis and develop effective gastric cancer treatments.

Reference

Recently, with advances in next-generation sequencing (NGS) technology, analysis of genomic and transcriptomic alterations in gastric cancer patients has been used to develop innovative methods for diagnosis and treatment of gastric cancer (3, 4). Molecular drivers of gastric cancer have been suggested through NGS-based genomic and transcriptomic analyses of mutation, deletion, amplification, fusion, and expression level. However, because gastric cancer is regarded as a heterogeneous and complex disease, the biological functions and clinical relevance of the candidate driver genes should be scrutinized for their applications in gastric cancer therapy.

GRP78 (glucose-regulated protein 78 kDa) is a well-known therapeutic target gene that is highly activated in various cancers, including gastric cancer (5–8). GRP78, also known as HSPA5 (heat-shock protein alpha 5), is a chaperone involved in protein folding in the endoplasmic reticulum (ER) and increases cell survival against apoptotic stresses, including anticancer drugs, by interacting with proapoptotic factors such as caspase-7 and BIK (9–11). In cancer, GRP78 is often relocalized to the plasma membrane (12, 13). Cell surface GRP78 activates AKT signaling through interaction with PI3K, thereby promoting tumorigenesis (14, 15). GRP78 inhibitors, such as small molecules and specific binding peptides, cause growth inhibition and apoptosis of cancer cells. Therefore, more effective drugs targeting GRP78 have been developed for cancer therapy (12, 16, 17). GRP78 has also been investigated and suggested as a biomarker and therapeutic target for gastric cancer therapy (18–20).

KIAA1324 gene, also known as EIG121 (estrogen-induced gene 121), encodes a 1,013 amino acid (a.a.) transmembrane protein that is highly conserved among species (21). The correlation between KIAA1324 expression and prognosis in endometrial, ovarian, and pancreatic cancer patients was
previously reported (21–23), but the role of KIAA1324 in gastric cancer has not been investigated yet. Deng and colleagues demonstrated that KIAA1324 localizes at the plasma membrane and endomembranes and is involved in autophagy (24). However, the biological functions of KIAA1324 are still poorly understood.

Here, we identified KIAA1324 as a candidate gastric tumor suppressor based on our transcriptome sequencing data from the tissues of gastric cancer patients. To investigate the potential tumor suppressive role of KIAA1324 in gastric cancer, we analyzed the correlation between KIAA1324 expression and gastric cancer patient prognosis and evaluated the tumorigenic abilities of gastric cancer cells expressing exogenous KIAA1324 using in vitro and in vivo assays. We observed that low KIAA1324 levels were associated with poor prognosis in gastric cancer patients. KIAA1324 inhibited growth, invasiveness, and tumorigenic activity of gastric cancer cells and induced apoptosis by blocking the oncogenic activities of GRP78. Taken together, our data suggest a novel role of KIAA1324 as a tumor suppressor and a prognostic indicator in gastric cancer.

Materials and Methods

Primary gastric cancer tissues

Fifty pairs of gastric cancer and normal matched control tissues were obtained from the gastric cancer depository of the Gastrointestinal Division in Department of Surgery at Seoul National University Hospital (Seoul, Republic of Korea). The Institutional Review Board of the Seoul National University Hospital approved management of the tissue depository and use of the tissues (IRB no. H-0806-072-248).

Cell culture and transfection

Human gastric cancer cell lines, MKN28, AGS, and SNU16 were obtained from Korean Cell Line Bank, authenticated by short tandem repeat profiling. These cell lines were expended and used within 10 passages. The cells were maintained in RPMI1640 containing 25 mmol/L HEPES (WelGENE) supplemented with 10% FBS (WelGENE), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (WelGENE). 293T cells were maintained in DMEM (WelGENE) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. FuGENE6 (Roche Applied Science) was used as a transfection reagent.

Establishment of gastric cancer cells expressing the KIAA1324 gene or KIAA1324 shRNA

We established MKN28 and AGS cell lines expressing KIAA1324 in a doxycycline-dependent manner (tet-on) using a retroviral system. SNU16 cell lines stably expressing control or KIAA1324 shRNA were generated as described previously (25). To establish MKN28 and AGS cell lines expressing KIAA1324 in a doxycycline-dependent manner (tet-on), the human KIAA1324 gene was cloned into pCMV-3HA vector (Clontech). For virus generation, 3HA-tagged KIAA1324 gene was inserted into a retroviral vector, pRETROx (Clontech). Viruses containing KIAA1324 gene or tetracycline response activator gene were produced according to the manufacturer’s protocol (Cell Biolabs, Inc.). MKN28 and AGS tet-on KIAA1324 cells were generated by infection with these viruses and selected by 2 µg/mL G418 and 2 µg/mL puromycin. For KIAA1324 knockdown, lentiviral constructs containing KIAA1324 shRNA (TRCN0000263310 and TRCN0000263310) were purchased from Sigma. SNU16 cell lines stably expressing control or KIAA1324 shRNA were generated by lentiviral infection and selected with 2 µg/mL puromycin.

Annexin V–positive cell population analysis

Annexin V staining was performed with BD Pharmingen Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s protocol. Cells were trypsinized and washed twice with PBS. The cells were incubated in binding buffer containing Annexin V-FTC and propidium iodide (PI). Stained cells were analyzed by flow cytometry using CELLQUEST program (Becton Dickinson).

Statistical analyses

All quantitative data are expressed as the mean ± SD. Kaplan–Meier analysis and Pearson χ² tests were performed using SPSS version 21.0 statistical software (IBM SPSS). Student t tests and one-way ANOVA were conducted using GraphPad Prism version 5 (GraphPad Software Inc.). P < 0.05 was considered statistically significant.

Results

KIAA1324 expression is suppressed in most gastric cancers

To identify novel gastric cancer–related genes, we analyzed transcriptome sequencing data acquired from 16 paired normal and tumor tissues of gastric cancer patients and 18 gastric cancer cell lines (26) and sorted differentially expressed genes (DEG) between gastric cancer and normal tissues (P < 0.01 with >2-fold change; Fig. 1A). Among the DEGs, we focused on KIAA genes, which were initially identified through the Kazusa cDNA project, because unknown genes often provide new insight into understanding cancer, and most of KIAA genes have remained functionally uncharacterized (27). We confirmed that expression patterns of the selected KIAA genes in gastric cancer cell lines were similar to those in primary tumor tissues (Fig. 1A). In particular, our data showed upregulation of KIAA1524, which is also known as CIP2A (cancerous inhibitor of protein phosphatase 2A) and was reported as an oncogene in gastric cancer (28). However, other KIAA genes have been poorly investigated, especially in gastric cancer. In this study, we focused our attention on the role of KIAA1324, which is the most significant downregulated KIAA gene in this set of gastric tissues and cancer cell lines. Suppressed KIAA1324 expression in most tumor tissues and gastric cancer cell lines was further validated using quantitative reverse transcription PCR (qRT-PCR; Supplementary Fig. S1). We also examined KIAA1324 expression in paired tissues of additional gastric cancer patients using qRT-PCR. As expected, KIAA1324 expression was significantly suppressed in cancer tissues (P < 0.0005; Fig. 1B). Paired comparison analysis of the tissues showed that 78% of patients had low levels of KIAA1324 at the tumor site compared with the normal region (Fig. 1C). These results were further supported by public microarray data (GSE13861; ref. 29), which showed low KIAA1324 expression in gastric tumor tissues (P = 0.0002; Fig. 1D).

The loss of gene expression is mainly caused by genetic alteration or epigenetic modification. Therefore, to investigate the mechanism by which KIAA1324 is regulated in gastric cancer cell lines, we first examined the correlation between gene copy number variation (CNV) and transcription level using CCLE database analysis (Supplementary Fig. S2). Copy number of KIAA1324
gene in 29 gastric cancer cell lines was barely changed and KIAA1324 expression was low in most gastric cancer cell lines. This result suggested that suppressed KIAA1324 expression is independent of copy number variation (CNV). Next, we explored whether KIAA1324 expression was regulated epigenetically using decitabine, a DNA methylation inhibitor, and MS-275, a synthetic histone deacetylase inhibitor. While MS-275 treatment restored KIAA1324 transcription in these gastric cancer cell lines with negligible expression, decitabine did not influence KIAA1324 expression (Fig. 1E). Combination treatment with MS-275 and decitabine in MKN1 cells enhanced restoration of KIAA1324 transcription compared with MS-275 treatment alone, but not in MKN28 and SNU638 cells. It has been known that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30). Our data suggest that densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones, and these two epigenetic processes have been dynamically linked (30).

Low levels of KIAA1324 are associated with poor prognosis in gastric cancer patients

To evaluate the clinical impact of KIAA1324, we performed a gastric tumor tissue microarray using the anti-KIAA1324 antibody and tumor tissues from 428 patients. As shown in Fig. 2A, the tumor tissues were classified into four groups (negative, weak, moderate, or strong) according to KIAA1324 expression. On the basis of this classification, we analyzed the cumulative survival rate of gastric cancer patients who provided the tumor tissues using the Kaplan–Meier test (Fig. 2B). We found that lower KIAA1324 expression was correlated with reduced survival rates of patients ($P < 0.001$). We also analyzed the relationship between KIAA1324 expression and clinicopathologic features (Fig. 2C). Patients aged 65 years or younger had lower KIAA1324 expression compared with older patients ($P = 0.038$). However, KIAA1324 expression was not associated with gender ($P = 0.389$). KIAA1324 expression was negatively correlated with tumor invasion ($P = 0.001$), pTNM stage ($P < 0.001$), lymph node metastasis...
KIAA1324 expression was correlated with poor prognosis in 428 gastric cancer patients. A, representative immunostaining images of gastric cancer tissues classified according to KIAA1324 expression (negative, weak, moderate, or strong). B and C, cumulative survival rate (P < 0.001; B) and clinicopathologic features (C) of 428 patients who were categorized into the four groups were investigated using tissue microarray and patient information. The Kaplan-Meier method and Pearson χ² tests were used for survival analysis and statistical analyses, respectively.

(KIAA1324 expression in tumors was verified by RT-PCR (Fig. 3I). These data demonstrate that KIAA1324 inhibits the tumorigenic activity of gastric cancer cells in vivo and the development of preformed tumors.

KIAA1324 inhibits in vivo tumor formation of gastric cancer cells

To investigate a possible role of KIAA1324 as a tumor suppressor in gastric cancer, we examined the effect of KIAA1324 on in vivo tumorigenesis of gastric cancer cells. For the xenograft assay, we generated stable MKN28 gastric cancer cell lines with tetracycline-inducible (tet-on) luciferase (Luc) or KIAA1324 expression. Luciferase was used as a control. We injected the MKN28 cells subcutaneously into mice after inducing KIAA1324 expression with doxycycline, a tetracycline analog, and observed tumor formation (Fig. 3A). Induction of KIAA1324 dramatically decreased the tumor formation ability of MKN28 cells as demonstrated by significantly reduced tumor sizes and weights at the time of harvest (P < 0.0005; Fig. 3B–D). Next, we investigated whether KIAA1324 affects the development of preformed tumors. Three weeks after subcutaneous injection of MKN28 cells with tet-on Luc or KIAA1324, the mice were fed water containing doxycycline every other day for a month (up to 7 weeks) to induce KIAA1324 expression (Fig. 3E). Tumor formation was observed within 3 weeks after injection. We measured tumor sizes weekly and obtained tumor weights at the time of harvest (Fig. 3E–H). The results showed that KIAA1324 induction significantly reduced the size (P < 0.0005) and weight (P < 0.001) of preformed tumors.

KIAA1324 expression in tumors was verified by RT-PCR (Fig. 3I). These data demonstrate that KIAA1324 inhibits the tumorigenic activity of gastric cancer cells in vivo and the development of preformed tumors.

KIAA1324 suppresses growth, invasion, and drug resistance of gastric cancer cells

We further investigated whether KIAA1324 influences the characteristic features of gastric cancer cells, including proliferation, invasiveness, and drug resistance. In addition to MKN28 cells, we established stable AGS gastric cancer cell lines with tet-on Luc or KIAA1324. The proliferation assay showed that KIAA1324 remarkably inhibited growth of MKN28 and AGS cells (Fig. 4A). KIAA1324 also suppressed anchorage-dependent and -independent colony forming activities of gastric cancer cells (Fig. 4B and C). To examine the effect of KIAA1324 on the invasiveness of gastric cancer cells, we assessed the migration and invasion ability of MKN28 and AGS cells expressing KIAA1324. As shown in Fig. 4D and E, KIAA1324 significantly reduced the migration and invasion of gastric cancer cells. We next explored whether KIAA1324 regulates the drug resistance of gastric cancer cells by treating MKN28 and AGS cells expressing KIAA1324 with anticancer drugs such as cisplatin and etoposide (Fig. 4F). Cells expressing KIAA1324 showed decreased cell viability in presence of cisplatin or etoposide compared with control cells. In addition, cisplatin- and etoposide-mediated apoptosis were increased in KIAA1324-expressing MKN28 cells (Supplementary Fig. S3). These results suggested that KIAA1324 caused the cells to become more sensitive to anticancer drugs. To further investigate whether
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the loss of KIAA1324 influences proliferation and drug resistance in SNU16 gastric cancer cells with KIAA1324 expression, we made an SNU16 cell line stably expressing KIAA1324 shRNA using a lentiviral system. KIAA1324 knockdown was confirmed by qRT-PCR (Fig. 4G). Although KIAA1324 knockdown did not dramatically affect SNU16 cell proliferation, it markedly enhanced cisplatin resistance (Fig. 4H and I). Moreover, loss of KIAA1324 decreased cisplatin- and staurosporine-induced apoptosis (Fig. 4J and Supplementary Fig. S4). Taken together, our data demonstrate that KIAA1324 inhibits the growth, invasiveness, and drug resistance of gastric cancer cells.

Ectopic expression of KIAA1324 induces apoptosis of gastric cancer cells

To examine whether KIAA1324 induces apoptosis of gastric cancer cells, we performed annexin V staining and flow cytometry analysis (Fig. 5A). Induction of KIAA1324 expression increased the annexin V-positive cell population, indicating that KIAA1324 induced apoptosis in MKN28 and AGS cells. We also confirmed KIAA1324-mediated apoptosis using a TUNEL assay (Fig. 5B). Next, we examined the expression of apoptosis markers using immunoblotting and RT-PCR. We verified activation of caspase-3, an apoptosis effector caspase protein, in gastric cancer cells expressing KIAA1324 by detecting cleavage of caspase-3 (Fig. 5C). Interestingly, expression of proapoptotic genes, such as BAX and BIM, were also increased by KIAA1324 (Fig. 5D). In addition, we investigated whether KIAA1324 affects cell-cycle distribution. KIAA1324 did not have a considerable effect on cell cycle, while inducing apoptosis (Supplementary Fig. S5). These results suggest that KIAA1324 exerts main effect on apoptosis. Because it has been previously mentioned that KIAA1324-mediated apoptosis might occur through excessive autophagy (24), we examined the expression of LC3B, an autophagy marker (Supplementary Fig. S6). However, we observed no significant difference between control and KIAA1324-expressing cells. In summary, our data suggest that KIAA1324 induces apoptosis through activation of a caspase cascade rather than autophagy.

The transmembrane domain of KIAA1324 is important for KIAA1324-mediated apoptosis

It has been reported that KIAA1324 is mainly localized in the membrane fraction, and deletion of its transmembrane domain (TM) limits its localization to the cytosol (24). To explore the role of TM in KIAA1324-induced apoptosis, we generated MKN28 and AGS cells with a tet-on TM-deleted mutant of KIAA1324 (KIAA1324 ΔTM). We confirmed expression of KIAA1324 ΔTM...
using immunoblotting (Fig. 6A and B). As shown in Fig. 6C, TM deletion resulted in defective cellular localization of KIAA1324. KIAA1324 ATM neither increased the annexin V–positive cell population nor activated caspase-3 (Fig. 6D and E). These results indicate that the TM of KIAA1324 is responsible for its ability to induce apoptosis as well as its cellular localization.

To further examine which region of KIAA1324, besides TM, is required for KIAA1324-mediated apoptosis, we analyzed apoptosis in cells expressing KIAA1324 mutants with deletion of the N-terminal side (ΔN) or C-terminal side (ΔC) of the TM. KIAA1324 ΔC exhibited similar cellular localization and apoptosis induction as wild-type KIAA1324 (Supplementary Fig. S7), whereas KIAA1324 ΔN did not induce apoptosis even though its localization was similar to wild-type KIAA1324 (Supplementary Fig. S8). To find more specific domain that is important for KIAA1324-mediated apoptosis, we examined the effects of a.a. 304–931 (ΔC1) and a.a. 657–931 (ΔC2) of KIAA1324 on apoptosis induction. While KIAA1324 ΔC induced apoptosis, ΔC1 and ΔC2 did not have any effect in KIAA1324-mediated apoptosis (Supplementary Fig. S9). These results demonstrate that KIAA1324 induces apoptosis of gastric cancer cells through the TM and the a.a. 1–303 region, suggesting that its cellular localization and the

Figure 4.
KIAA1324 inhibited proliferation, invasiveness, and drug resistance of gastric cancer cells. A, doxycycline-induced KIAA1324 expression in MKN28 and AGS cells harboring tet-on KIAA1324 was verified by immunoblotting. Proliferation of MKN28 and AGS cells harboring tet-on Luc or KIAA1324 was evaluated daily in the presence or absence of 1 µg/mL doxycycline. B, representative images of methylene blue–stained colonies of MKN28 and AGS cells expressing Luc or KIAA1324. Relative colony forming unit (CFU) was calculated by dividing the colony number of doxycycline-untreated cells by that of doxycycline-treated cells. C, soft agar colony forming assay was performed to investigate the effect of KIAA1324 on anchorage-independent colony formation of MKN28. Migration (D) and invasion (E) abilities of MKN28 and AGS cells expressing Luc or KIAA1324 were measured using Transwell migration and invasion assay, respectively. The relative migration or invasion rates were calculated by dividing cell number of doxycycline-untreated cells divided by that of doxycycline-treated cells. F, cell viability of MKN28 and AGS cells harboring tet-on KIAA1324 was measured 24 hours after treatment with 25 µmol/L cisplatin or 25 µmol/L etoposide in the absence or presence of 1 µg/mL doxycycline. G, KIAA1324 knockdown in SNU16 cells expressing KIAA1324 shRNA was examined using qRT-PCR. H, growth of SNU16 cells expressing control or KIAA1324 shRNA was measured daily. Cell viability (I) and caspase-3 activation (J) of SNU16 cells expressing control or KIAA1324 shRNA were evaluated 24 hours after treatment with 25 µmol/L cisplatin by cell counts and immunoblotting, respectively. *, P < 0.005; ***, P < 0.0005.
a.a. 1–303 region are crucial for KIAA1324-mediated apoptosis in gastric cancer cells.

**GRP78 oncprotein is identified as a KIAA1324-binding partner**

To elucidate the regulatory mechanism of KIAA1324-induced apoptosis, we identified KIAA1324-specific binding partners through protein interaction analysis using a formaldehyde cross-linking method (Fig. 7A; ref. 31). We found GRP78 as a KIAA1324-binding partner and validated the interaction between KIAA1324 and GRP78 (Fig. 7B). Immuno fluorescence analysis also demonstrated that KIAA1324 colocalized with GRP78 (Fig. 7C). Because GRP78 is predominantly localized in the ER, we investigated whether KIAA1324 also exists in the ER by examining colocalization with PDI, an ER marker. As expected, KIAA1324 was found in the ER (Supplementary Fig. S10). KIAA1324 was also located in the cell membrane in accordance with the previous report (24).

To determine the binding region of GRP78 with KIAA1324, we examined intersections between KIAA1324 and various domain-deleted mutants of GRP78 (Fig. 7D–F). Immunoprecipitation assays showed that a.a. 1–80 of GRP78, which contains Thr37, an ATP-binding site (32), was required for the GRP78–KIAA1324 interaction. In addition, analysis of the interactions between GRP78 and domain-deleted mutants of KIAA1324 demonstrated that a.a. 1–303 of KIAA1324 was responsible for the interaction with GRP78 (Fig. 7G and H and Supplementary Fig. S11).

**KIAA1324 inhibits the oncogenic activity of GRP78**

GRP78 increases cancer cell survival by exerting antiapoptotic activity by interacting with caspase-7 in the ER and by activating pro-proliferative PI3K/AKT signaling in the cell membrane. Given that inhibition of GRP78 induces apoptosis and increases anticancer drug sensitivity in gastric cancer cells (16, 20), we investigated the effect of siRNA-mediated GRP78 knockdown on MKN28 cells. As expected, we found that GRP78 knockdown induced apoptosis of MKN28 cells and decreased AKT phosphorylation (Supplementary Fig. S12).

It has been reported that Thr37 of GRP78 is important for ATP-induced conformational change, which is critical for the interaction of GRP78 with its binding partners (32), and the N-terminal side of the GRP78 ATPase domain is required for AKT activation (15). Therefore, because KIAA1324 interacted with N-terminal region of GRP78, we first investigated whether KIAA1324 affects GRP78 binding to caspase-7. Indeed, KIAA1324 wild-type and ΔC mutant blocked the interaction between GRP78 and caspase-7 and induced cleavage of caspase-7 and PARP (Fig. 7I). However, KIAA1324 ΔTM and N-terminal region (N) did not affect GRP78 binding to caspase-7 despite their interaction with GRP78. This result suggests that the TM of KIAA1324 is required for KIAA1324 to interfere with the interaction between GRP78 and caspase-7 despite their interaction with GRP78. This result suggests that the TM of KIAA1324 is required for KIAA1324 to interfere with the interaction between GRP78 and caspase-7. Next, we investigated whether KIAA1324-mediated regulation of GRP78 affects AKT activation by examining AKT phosphorylation (Fig. 7I). The result showed reduction of AKT phosphorylation in MKN28 cells expressing KIAA1324. Taken together, these data
demonstrate that KIAA1324 not only inhibits the interaction between GRP78 and caspase-7, but also may regulate GRP78-mediated AKT activation, suggesting that KIAA1324 may exert antitumor activity by suppressing the oncogenic activities of GRP78 (Supplementary Fig. S13).

**Discussion**

To date, a number of tumor suppressor genes have been identified and investigated for their function in tumorigenesis. However, many potential tumor suppressor genes remain unknown and uncharacterized. Recently, analysis of genetic alterations and transcriptome changes in cancer tissues and cell lines using NGS has become a promising method to identify candidate tumor suppressor genes (3, 33). Moreover, studying the biological functions of these genes improves our understanding of the underlying mechanisms of carcinogenesis and aids in cancer prevention and therapeutics. In the current study, KIAA1324 was identified as a novel tumor suppressor that is downregulated in human primary gastric cancer tissues and cell lines through total mRNA sequencing analysis (Fig. 1). CNV analysis and an epigenetic modulation assay of gastric cancer cell lines showed that suppression of KIAA1324 expression in gastric cancer cells is caused by epigenetic regulation rather than genetic alteration (Fig. 1E and Supplementary Fig. S2). A study of the clinical impact of KIAA1324 demonstrated that KIAA1324 can be used as a biomarker for prognostic prediction of gastric cancer (Fig. 2). Furthermore, investigation of the effects of KIAA1324 on proliferation, tumorigenic activity, and apoptosis of gastric cancer cells indicated that KIAA1324 may function as a gastric tumor suppressor (Figs. 3, 4, 5). In particular, induction of KIAA1324 expression in preformed tumors significantly reduced tumor size (Fig. 3E–H). This result suggests that, with remarkable recent developments in tumor-specific drug or gene delivery system for cancer therapy (34, 35), application of KIAA1324 gene delivery or a KIAA1324-inducible drug release system specifically targeting gastric tumors can be a feasible strategy for gastric cancer therapy.

GRP78 has been regarded as a promising therapeutic target for cancer therapy. As GRP78 enhances cancer cell survival by protecting cancer cells from apoptotic stresses such as anticancer drugs, targeting GRP78 increases efficacy of cancer treatment (17). In cancer, the role of GRP78 in the cell membrane as well as the ER have drawn interest because increased cell surface GRP78 is detected in various cancers. GRP78 is principally localized in the ER lumen; however, it also has been demonstrated that GRP78 has putative transmembrane domains and localizes at the cellular membrane as well as the ER membrane (9, 13). Cell surface GRP78 has been reported to regulate proliferation, migration,
and invasion of cancer cells via regulation of various cellular signaling pathways, including the TGFβ and AKT signal pathways (36–39). Peptides targeting cell surface GRP78 have suppressed tumor growth and invasion, suggesting that GRP78-targeting peptides could be a therapeutic strategy for patients with cell surface GRP78-positive tumors (12, 40). We demonstrated that KIAA1324 physically interacted with the N-terminal domain of GRP78 through its N-terminal region and might regulate GRP78-mediated AKT activation (Fig. 7). A domain prediction program indicated that the N-terminal region of KIAA1324 may be an extracellular domain, and a previous report and our immunofluorescence data showed that KIAA1324 localized at cell membrane (24). Taken together, these findings suggest that KIAA1324 may also modulate cell surface GRP78 extracellularly through its N-terminal domain. On the basis of our findings, identification of the critical motif of KIAA1324, which is necessary for the interaction with GRP78, may be a platform for the development of GRP78-targeting KIAA1324 peptides as anticancer therapeutic agents.

In cancer, the role of GRP78 as a receptor at the cell surface depends on extracellular ligands. Alpha-2 macroglobulin interacts with the N-terminal region of cell surface GRP78 and activates AKT signaling, leading to increased cell proliferation (41). However, Kringle 5 induces caspase-7 activation by binding to cell surface GRP78 (40). Par-4 also induces apoptosis via interaction with cell surface GRP78 and activation of the FADD-caspase 8–caspase-3 pathway (42). In this study, we demonstrated that through interaction with GRP78, KIAA1324 induced activation of caspase-3 and -7 and decreased AKT signaling. Considering previous reports, our data suggest that KIAA1324 may release caspase-7 from GRP78 in the ER, activate the FADD-caspase 8–caspase-3 pathway in the cell membrane, and block alpha-2 macroglobulin from binding to cell surface GRP78, thereby inducing apoptosis.
Deng and colleagues reported that KIAA1324 was predominantly in the membrane fraction and colocalized with autophagosome markers, suggesting that it is involved in autophagy (24). They also suggested that the observed KIAA1324-induced apoptosis in 293T cells occurred due to excessive autophagy. However, autophagic death and apoptosis are regarded as different types of cell death (43, 44). Furthermore, we could not observe KIAA1324-mediated autophagy in gastric cancer cells (Supplementary Fig S6). In our study, KIAA1324 induced cell death via an apoptotic mechanism. We found that KIAA1324 blocked the interaction between GRP78 and proapoptotic caspase-7, suggesting that KIAA1324 induces apoptosis of gastric cancer cells by inhibiting the antiapoptotic activity of GRP78.

It has been reported that transmembrane proteins in the ER, such as TMEM166 and TMEM214, are involved in the induction of apoptosis (45, 46). TMEM166 contains a single transmembrane domain and induces both autophagy and apoptosis. Compared with normal tissues, TMEM166 is downregulated in gastric adenocarcinoma (47). Adenovirus-mediated introduction of TMEM166 suppressed tumor growth through autophagy and apoptosis (48). TMEM214, which contains two transmembrane domains and is localized to the outer membrane of the ER, regulates ER stress-induced apoptosis through activation of caspase-4 (46). Here, we observed that KIAA1324, a transmembrane protein, induced apoptosis in the ER, and TM deletion abolished its apoptotic activity even though the TM deletion mutant bound to GRP78. It is possible that these transmembrane proteins interact with each other at the ER membrane to induce apoptosis by regulating GRP78. Further investigation of the interaction among these proteins will provide a better understanding of KIAA1324-mediated apoptosis in cancer.

AKT signaling plays a key role in various cellular processes including proliferation, survival, metabolism, differentiation, and apoptosis (49). Loss of AKT inhibitors such as PTEN and SHIP or upregulation of AKT activators such as GRP78 and Src induces dysregulation of AKT activation and has been implicated in carcinogenesis. In the current study, we observed that KIAA1324 reduced AKT phosphorylation in MKN28 cells. This phenomenon may be attributed to KIAA1324-mediated inhibition of GRP78 activity. However, we cannot exclude the possibilities that KIAA1324 regulates AKT signaling directly or through interaction with other regulators of AKT. Therefore, further studies to explore the possibilities will support tumor suppressive role of KIAA1324 as a negative regulator of AKT.

The role of KIAA1324 in cancer has been evaluated only in endometrial, pancreatic, and ovarian cancer to date (21–23). In type I endometrial cancer, KIAA1324 expression is higher at early stage than that of benign tumors, but reduced in high grade and stage endometrial carcinoma. In addition, KIAA1324 is downregulated in type II endometrial cancer, which is more aggressive than type I. In pancreatic cancer, KIAA1324 is also highly expressed in early-stage tumor, but its expression is decreased in advanced cancer. High KIAA1324 expression in endometrial and pancreatic carcinoma is correlated with favorable prognosis in cancer patients. However, in high-grade serous carcinoma of the ovary/peritoneum, high expression of ERα and KIAA1324 is associated with poor survival in cancer patients. This indicates that KIAA1324 may play different roles in various types of cancers. In our study, we provide evidence that supports a tumor suppressive role of KIAA1324 in gastric cancer through induction of apoptosis. Our study may lead to further investigation of the function of KIAA1324 in other cancers.

In conclusion, our study demonstrated that KIAA1324 was epigenetically downregulated in gastric cancer and positively correlated with prognosis of gastric cancer patients. We also revealed that KIAA1324 suppressed growth of gastric cancer cells and tumors by inhibiting the oncogenic activity of GRP78. Taken together, we suggest KIAA1324 as a novel gastric tumor suppressor and provide a new insight for the application of KIAA1324 in the diagnosis and treatment of gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J.M. Kang, S. Park, H.-K. Yang, S.-J. Kim
Development of methodology: J.M. Kang, S.-J. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Kang, S. Park, S.J. Kim, H. Kim, B. Lee, W.H. Kim, S.-J. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Kang, S. Park, B. Lee, J. Kim, S.T. Kim, W.H. Kim
Writing, review, and/or revision of the manuscript: J.M. Kang, S. Park, S.J. Kim, H. Kim, B. Lee, J. Kim, H.-K. Yang, W.H. Kim, S.-J. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Kang, S. Park, B. Lee, S.T. Kim
Study supervision: S.-J. Kim

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