Associations between mutations of the cell cycle checkpoint kinase 2 gene and gastric carcinogenesis

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Abstract. Gastric cancer is the most common malignant tumor of the digestive system. The etiology of gastric cancer is complex, and susceptibility at the genetic level remains to be fully elucidated in genetic investigations. In the present study, mutations of the cell cycle checkpoint kinase 2 (CHEK2) gene and its association with gastric cancer were examined. Reverse transcription-quantitative polymerase chain reaction technology was used to detect the expression of CHEK2 and it was found that the expression of CHEK2 was low in gastric cancer. Using sequencing analysis, it was found that the low expression level of CHEK2 was associated with expression of its mutation. The present study also established a CHEK2-overexpressing mutant and confirmed that CHEK2 promoted gastric cancer cell proliferation. Overexpression of the CHEK2 mutation was confirmed to promote cancer cell migration and invasion. Furthermore, western blot analysis results revealed that overexpression of the CHEK2 mutation downregulated E-cadherin and upregulated vimentin expression, indicating the mechanism underlying the altered biological behavior. These results suggested that there was a correlation between mutation of the CHEK2 gene and gastric cancer, and provided an experimental basis for antitumor drug investigation and development according to its mutation target.

Introduction

Gastric carcinoma is one of the fourth most common types of malignant tumor worldwide (1-3). As the most common type of malignant tumor of the digestive system, gastric cancer has been confirmed as the second leading cause of cancer-associated mortality worldwide from statistical data of mortality rates (4,5). The etiology of gastric cancer is complex, involving various factors, including age, gender, socioeconomic status, state of health and environmental risk factors (6-8). All these factors occur in different stages, targeting different genes and regulatory factors, and causing changes in the structure and expression level of associated genes, eventually leading to the occurrence and development of gastric cancer due to the combination of these genes and regulatory factors (9-12). Surgery is a primary treatment option for gastric cancer, and is also the only option expected to cure this type of cancer (13). Due to a lack of characteristic clinical manifestations in early gastric cancer, diagnosis is difficult, therefore, the majority of patients have already developed advanced gastric cancer, which is passed the optimal stage for surgery (14,15). The treatment efficacy for advanced gastric carcinoma is poor and the overall prognosis is poor. Therefore, identifying molecular diagnostic indices and effective therapeutic targets at the tumor gene level have become a novel area of interest in gastric cancer.

Cell cycle checkpoint kinase 2 (CHEK2) has been identified as a type of serine/threonine protein kinase, located in chromosome 22 q12.1 of humans and yeast. It has been confirmed as an important mediator of the DNA damage response pathway, and as a susceptibility gene in several types of cancer (16,17). As a tumor suppressor gene, CHEK2 is vital for the induction of cell cycle arrest and cell apoptosis following DNA damage. When CHEK2 mutation occurs, the coding kinase is inactivated and the damaged DNA cannot repair, following which abnormal DNA replicates uncontrollably, leading to the cancer (18,19). As primary forms of mutations in the malignant tumor, missense mutations and truncated mutants lead to a significant decrease or even the complete loss of CHEK2 kinase activity (20,21). For CHEK2, the four gene mutation sites, 1100delc, IVS2G>A, del5395 and 1157T, have been identified (22). Among these, 1100delc, IVS2G>A and del5395 are truncated mutations, which can lead to the truncation of premature proteins. 1157T is a missense mutation, consisting of an isoleucine for threonine replacement (23).

Previous studies have found that the CHEK2 gene is a multi-tumor susceptibility gene. In patients with breast cancer, prostate cancer, colon cancer and other types of malignant tumor, low frequency mutations were found in embryonic and somatic cells (24,25). In addition, mutation carriers are at increased risk of breast cancer, thyroid cancer, bladder cancer, ovarian cancer and colorectal cancer (26,27). Mutations in

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CHEK2 have been associated with cancer at several sites; CHEK2 mutations have been reported to predispose to gastric cancer, particularly to young-onset cases (28). At present, the specific role of the CHEK2 gene in gastric cancer and its mechanism remain to be fully elucidated, and the association between CHEK2 and gastric cancer requires extensive investigation.

In the present study, patients with gastric cancer and healthy individuals were assessed for gene mutations using polymerase chain reaction (PCR) analysis combined with a gene sequencing assay, in order to investigate the association between the CHEK2 gene and gastric cancer. The results confirmed that the expression of CHEK2 was low in gastric cancer and the low expression of CHEK2 was closely associated with expression of its mutation. Detailed investigations were performed on the mechanism of CHEK2 mutation, providing an experimental basis for the development of anticancer drugs contraposing its mutation targets.

Materials and methods

Collection of gastric cancer data. The collection and sorting of clinical data was performed for a total of 105 cases of gastric carcinoma using conventional paraffin-embedded tissue samples and fresh specimens from surgery between May 2013 and June 2014 at the Second Affiliated Hospital of Soochow University (Suzhou, China). Of the patients recruited, 63 were men, 42 were women and all were aged between 30 and 82 years (median, 62 years). None of the patients had received preoperative cancer treatment. All cases were diagnosed as gastric adenocarcinoma using conventional hematoxylin and eosin staining, which comprised 6 cases with well differentiated carcinoma, 25 cases of moderately differentiated carcinoma and 74 cases of poorly differentiated carcinoma. In addition, 62 cases exhibited lymph node metastases, whereas 43 cases showed no lymph node metastasis. No cases exhibited distant metastases. According to the Union for International Cancer Control installment standard P-TNM classification (revised in 1997) (29), of the 105 cases, there were: 2 cases in stage 0, 24 cases in stage I, 42 cases in stage II, 31 cases in stage III and 6 cases in stage IV. From all cases, normal gastric tissue was obtained from 5 cm away from the stomach tissue tumor lesions as negative control. The present study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University. Written informed consent was obtained from all patients.

Blood samples and genotyping. The fresh blood samples from each individual were anticoagulated with 3.8% sodium citrate, and cells from each sample were isolated by centrifugation (400 x g for 30 min at 22˚C) and frozen at -80˚C until use. The DNA extraction kit and paraffin-embedded tissue DNA extraction kit were from Tiangen Biotech Co., Ltd. (Beijing, China). The TaqMan MGB probe (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and primers were used for rs2236142 genotyping. Primer pairs were as follows: 1100delc forward, 5'-GCA AAA TTA AAT GTC CTA ACT TGC-3' and reverse, 5'-CCA GTC TGT GCA GCA ATG AA-3'; IVS2G>A forward, 5'-CTA CTG GTT TGG GAG GGA CA-3' and reverse, 5'-GA CCA AAT TAC CAG CTC TCC-3'; Del5395 forward, 5'-AGG TGC AGC ATC CTG TTC G-3' and reverse, 5'-AGG ATT GCT CCC AAT CAC TG-3'; 1157T forward, 5'-TTT CGG ATT TTC AGG GTA GG-3' and reverse, 5'-ATT CAA AGG ACG GCG TTT TC-3'. The PCR amplification procedure was as follows: Initial denaturation at 95˚C, 2 min; 33 cycles of 95˚C for 30 sec, 58˚C for 30 sec, 72˚C for 1 min. The genotyping experiments were repeated for 15% of the sample and the results were 100% consistent.

Cell culture. Normal gastric mucosa cells (GES1), and the gastric cancer cell lines BGC-823, SGC7901 and AGS were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured in 10% fetal bovine serum and penicillin/streptomycin DMEM medium (both from Gibco; Thermo Fisher Scientific, Inc.) in an incubator at 37˚C and 5% CO_2.

RNA isolation and cDNA generation. Total RNA from the gastric tissues and cell lines were isolated using a miRNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was generated using the stem-loop reverse transcript primer and Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA). RT-qPCR was performed on the QuantStudio 6 Flex Realtime PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling parameters were as follows: 2 min at 95˚C, followed by 45 cycles of 10 sec at 95˚C and 60 sec at 60˚C. The following primers were used: CHEK2 forward, 5'-CCC TCC CAA ACC AGT AGT TGT-3' and reverse, 5'-ACA GCC CCA TGG CAG CG-3'; GAPDH forward, 5'-GAG TCC ACT GGC GTC TTC-3, reverse, 5'-GTG CTA AGC AGT TGG T-3'. Human GAPDH served as an internal control. Data were analyzed with the $2^{-\Delta\Delta Cq}$ method (30).

Plasmid construction. For CHEK2 overexpression, the complete coding sequence of mutated CHEK2 (1100delc) was cloned into a pcDNA3 vector (Thermo Fisher Scientific, Inc.) to form the vector pCHEK2-Mut. The blank vector served as a control. Transfection was performed in 24-well plates ($2x10^5$ BGC-823 or SGC7901 cells in each well) with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h after transfection, the cells were used in the following experiments.

MTT assay. For the MTT assay, BGC-823 and SGC7901 cells ($1x10^5$) were seeded into 96-well plates, and transfected with pCHEK2-Mut or the negative control (blank pcDNA3) for 48 h. At 24, 48 and 72 h post-transfection, 10 μl MTT (0.5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the culture solution. The cells were incubated at 37˚C for 4 h and the medium was removed. To dissolve the formazan, 100 μl DMSO (Sigma-Aldrich; Merck KGaA) was added, and a Quant universal microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) was used to observe the absorbance at 570 nm (A570).

Cell proliferation assay. Cell proliferation was assessed using the colony formation experiment. Following transfection with pCHEK2-Mut or pcDNA3 as aforementioned, a total

Table I. Genotype analysis in gastric cancer tissue and blood samples.

		Genotype			
Gastric cancer sample	n	1100delc (%)	IVS2G>A (%)	Del5395 (%)	1157T (%)
Tissue	105	5 (4.7)	2 (1.9)	0 (0)	1 (0.9)
Blood	105	3 (2.8)	1 (0.9)	1 (0.9)	0 (0)

of 200 cells in the logarithmic phase were seeded in 6-cm culture dishes with culture medium, and the plates were incubated in a humidified atmosphere at 37˚C for 2 weeks. When microscopic colonies were observed, the culture medium was discarded and the cells were washed gently twice with PBS, fixed in 4% paraformaldehyde for 15 min and stained in Giemsa (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. The cells were air-dried, and the colony number of each group was counted under an optical microscope. The experiment was repeated three times.

Migration and invasion assay. At 48 h following pCHEK2-Mut or pcDNA3 transfection, the cells were used for a Transwell assay. For cell invasion analysis, a Transwell chamber with an 8.0-μm pore polycarbonate filter insert pre-coated with Matrigel was used. Cells ($5x10^4$) were suspended in 200 μl serum-free medium and the cell suspension was seeded in the upper chamber. The lower chamber was filled with 600 μl normal culture medium. The 24-well plates were incubated at 37˚C for 48 h, and non-traversed cells were subsequently wiped away with a cotton swab. Traversed cells on the lower side were stained with crystal violet for 20 min, and counted under an optical microscope (BH-2; Olympus Corporation, Tokyo, Japan). For cell migration analyses, the same procedures were performed without the use of Matrigel.

Western blot. Protein samples of cells were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and quantified using a bicinchoninic acid protein assay. The protein samples (0.1 mg) were separated by 10-12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The blot was blocked in 5% skimmed milk for 2 h at room temperature and subsequently incubated with the primary antibodies at 4˚C overnight for the detection of: E-cadherin (1:1,000; ab76055) and vimentin (1:1,000; ab8978) (both from Abcam, Cambridge, UK). GAPDH (1:5,000; ab8245; Abcam) served as an internal control. The membrane was subsequently incubated with the anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (1:5,000; ab131368) at room temperature for 1 h. The positive bands were visualized by Plus Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.), and the grey level of bands was analyzed using ImageJ 1.49 (National Institutes of Health, Bethesda, MD).

Statistical analysis. The data were presented as the mean ± standard deviation of at least three independent experiments. Student's t-test (two-tailed) was used. $P<0.05$ value was considered to indicate a statistically significant difference. Statistical analyses were performed using Graphpad 6.0 (GraphPad Software Inc., San Diego, CA).

Results

CHEK2 is expressed at low levels in gastric cancer tissues and gastric cancer cells. The present study detected the mRNA expression level of CHEK2 in 105 pairs of gastric cancer tissue specimens and normal adjacent tissues using RT-qPCR analysis. The results showed that the expression of CHEK2 in the gastric cancer group was significantly lower, compared with that in than normal group (Fig. 1A). Subsequently, the present study detected the mRNA expression level of CHEK2 in normal gastric mucosa cells (GES-1), and in BGC-823, SGC7901 and AGS gastric cancer cells using RT-qPCR analysis (Fig. 1B). The results were consistent with the results obtained in the tissue assays above. All these results suggest that CHEK2 was expressed at low levels in gastric cancer tissues and gastric cancer cells.

CHEK2 mutation is present in patients with gastric cancer. It has been shown that the genetic susceptibility of several types of malignant tumor is associated with CHEK2 gene mutations, including gastric cancer, breast cancer and prostate cancer (28,31,32). Whether the low expression of CHEK2 in gastric cancer is associated with its mutations required detailed investigation. In the present study, RNA was extracted from tissue samples and serum of from 105 cases of gastric cancer. The RNA was then reversed transcribed into cDNA for sequence alignment of the expression of CHEK2. The results (Table I) showed that the CHEK2 mutation was present in patients with gastric cancer, with 1100delc and IVS2G>A being the two predominant mutation forms.

CHEK2 (1100delc) promotes proliferation of gastric cancer cells. The present study subsequently examined the effects of mutant CHEK2 (1100delc) on gastric cancer cell vitality. The CHEK2 (1100delc) mutation plasmid, pCHEK2-Mut, was first constructed, and pCHEK2-Mut was transfected into the BGC-823 and SGC7901 gastric cancer cell lines. The effects of the mutant on gastric malignant proliferation were determined using MTT and clone formation assays. As shown in Fig. 2, the CHEK2 (1100delc) mutation promoted the proliferation and clone formation of the gastric cancer cells. In conclusion, the mutant CHEK2 (1100delc) promoted malignant gastric cancer cell proliferation.

CHEK2 (1100delc) promotes gastric cancer cell migration and invasion. In the subsequent experiments, Transwell

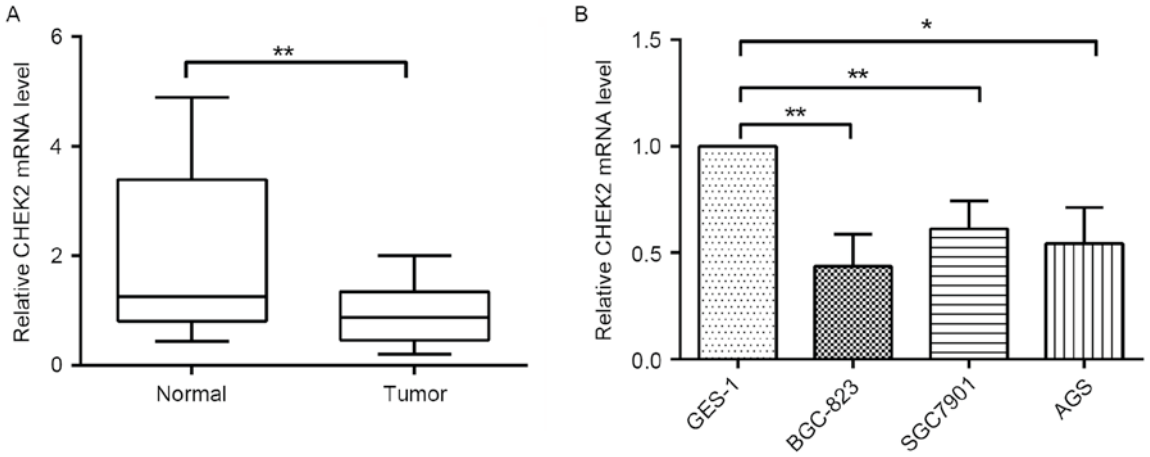

Figure 1. mRNA expression levels of CHEK2. The relative mRNA expression level of CHEK2 in (A) normal tissues, gastric tumor tissues, and (B) gastric cancer cells were detected using reverse transcription-quantitative polymerase chain reaction analysis. *P<0.05 and **P<0.01. CHEK2, cell cycle checkpoint kinase 2.

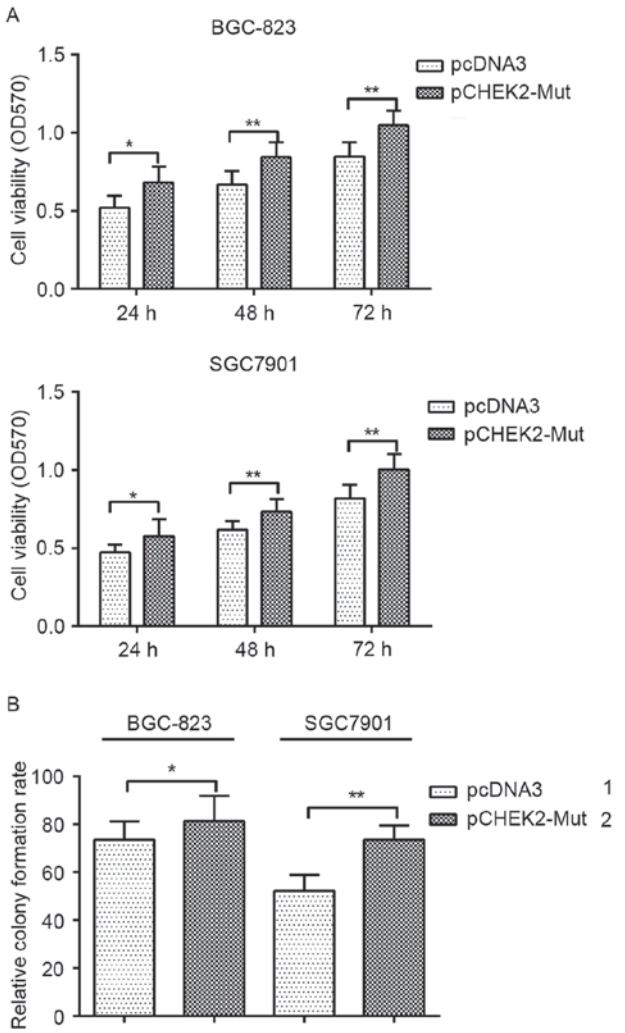

Figure 2 Effects of CHEK2 (1100delc) on gastric cancer cell proliferation. (A) Cell viability and (B) proliferation were respectively detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and colony formation assays *P<0.05 and **P<0.01. CHEK2, cell cycle checkpoint kinase 2; mut, mutant.

migration and invasion experiments were performed to examine the effects of mutant CHEK2 on gastric cancer cell migration and invasion. The results, as shown in Fig. 3, showed that transfection of BGC-823 and SGC-7901 gastric cancer cells with pCHEK2-Mut promoted the cell migration and invasion abilities.

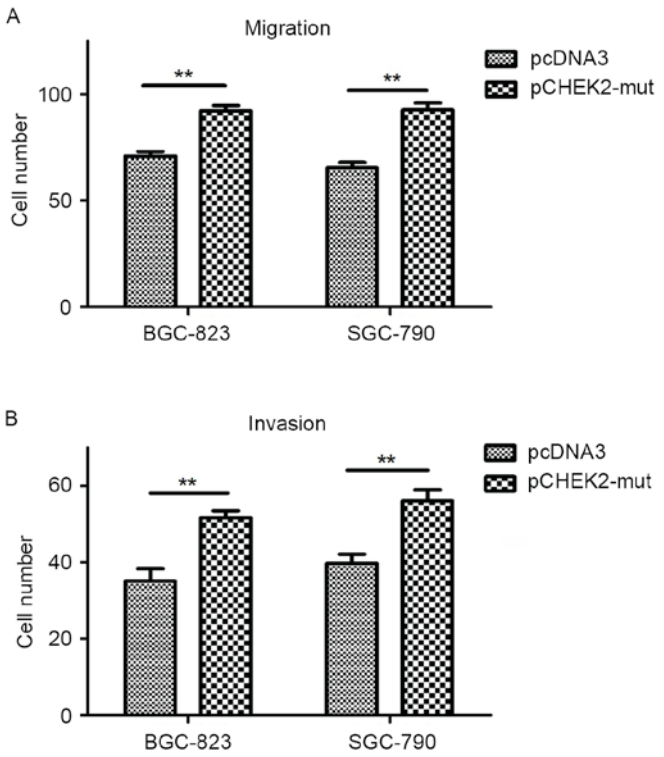

Figure 3. Effects of mutant (1100delc) on gastric cancer cell migration and invasion. (A) Migration and (B) invasion of BGC-823 and SGC-790 gastric cancer cells are shown. **P<0.01. CHEK2, cell cycle checkpoint kinase 2; mut, mutant.

Molecular mechanism of CHEK2 (1100delc) on gastric cancer. Based on the results described above, the present study examined the expression of tumor metastasis related proteins in BGC-823 and SGC-790 cells. Western blot analysis (Fig. 4) indicated that mutant CHEK2 (1100delc) downregulated E-cadherin expression, and upregulated vimentin expression.

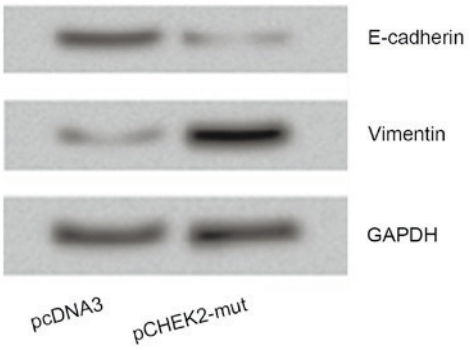

Figure 4. Epithelial-mesenchymal transition and cell cycle of gastric cancer cells. The expression of E-cadherin and vimentin in BGC-823 gastric cancer cell was detected by western blot analysis. CHEK2, cell cycle checkpoint kinase 2; mut, mutant.

Discussion

CHEK2 is a type of serine/threonine protein kinase, located in chromosome 22 q12.1 of humans and yeast, and has been reported to encode for a G2 checkpoint kinase, which is critical in DNA repair (32,33). As an important signal transducer of cellular responses to DNA damage, and a candidate tumor suppressor contributing to molecular pathogenesis of several types of human malignancy, germline mutations in CHEK2 (1100delC, IVS2+1G>A and I157T) have been confirmed to be associated with several types of cancer (34,35). Its mutation confers an increased risk of sporadic and hereditary disease, including breast cancer and prostate cancer (36,37).

CHEK2 is involved in DNA damage repair and protects the integrity of the genome. The reaction pathway is activated following DNA damage, leading to stagnation of the cell cycle, providing time for damage repair, and inducing gene transcription to facilitate repair at the same time (38). If DNA damage is unable to repair, the damaged cell can initiate apoptosis, and CHEK2 kinase may be involved in the DNA damage caused by cell apoptosis (39). Defects of the DNA damage response pathway leads to genomic instability and cancer susceptibility. In the study by Stolz *et al*, the CHEK2 gene was confirmed as being necessary in maintaining chromosomal stability in the process of mitosis, although this action was not associated with DNA damage (40). The abnormal CHEK2-mediated phosphorylation of BRCA1 can lead spindle assembly errors in the M phase of mitosis, thus inducing human cell chromosome instability, which is an important characteristic of tumor cells, and is important in the processes of tumor occurrence and development (41). Previous studies have shown that CHEK2 mutations predispose to gastric cancer, particularly young-onset cases (28), however, the association between CHEK2 and gastric cancer, and the underlying molecular mechanism require extensive investigation.

The present study preliminary discussed the role of CHEK2 in gastric cancer, and it was found that the expression of CHEK2 was low in gastric cancer tissues and gastric cancer cells using RT-qPCR analysis. In the following experiments, this was found to be due to mutation by examining 105 cases of gastric cancer tissues and serum RNA samples. Furthermore, the CHEK2 mutant was found to promote cell proliferation, migration and invasion in malignant gastric cancer.

Epithelial-mesenchymal transition (EMT) is a carcinogenesis-enabling process, and is important in the initiation of invasion and metastasis. N-cadherin is an EMT marker positively associated with the metastasis of tumor cells, whereas E-cadherin is a negative correlation marker (42). It is governed by several signal transduction pathways, which culminate in core transcription factors of the process (43). On investigating the mechanism of CHEK2 in gastric cancer, the present study demonstrated that mutant CHEK2 (1100delc) downregulated E-cadherin expression and upregulated vimentin expression, indicating the mutant CHEK2 (1100delc) promoted gastric cancer cell migration and invasion through regulation of EMT-related proteins.

Taken together, the observations in the present study revealed novel roles of CHEK2 in gastric cancer, provided novel information regarding molecular genetics and assisted in clarifying the pathogenesis, suggesting it is a potential target for developing therapeutics against gastric cancer. Specific elements of the follow-up mechanism in animal models and the signaling pathway require further validation through experiments.

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