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Research Paper

HERG K+ channels expression in gastric cancers and analysis of its regulation in tumor cell proliferation and apoptosis ☆

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Abstract

Objective: To investigate the expression of herg1 gene in tumor tissues from gastric carcinomas and gastric carcinoma cell lines, and study the relationship between HERG K+ channel expressions and tumor cell proliferation and apoptosis. **Methods:** RT-PCR and PCR assays were used to detect the expression of herg1 gene in 64 gastric carcinomas and the gastric cancer cell line SGC-7901. Blocking the HERG K+ channels was used to evaluate their effects on tumor cell proliferation and apoptosis. **Results:**The statistically significant expression of herg1 gene was detected in all the gastric cancers and SGC-7901 cells, but not in normal tissues. The HERG K+ channel blocker, E-4031, increased the cell population in G0/G1($P < 0.05$) and the number of apoptotic tumor cells($P < 0.05$). **Conclusion:** HERG K+ channels were expressed in all gastric carcinomas tested and these channels appear to modulate tumor cell proliferation and apoptosis.

Keywords: gastric carcinoma ; HERG K+ channel; herg 1 gene; proliferation; apoptosis

INTRODUCTION

Cancer development is a multistep process, which includes the alterations in cell growth and survival. The tumor cell proliferation plays an important role in the cancer growth and has become a topic of considerable interest to researchers.

Potassium channels are the most diverse class of plasma membrane ion channels, and this heterogeneity is reflected by the large variety of specific roles they exert in different cell types. Besides the regulation of excitability in nerve and muscle cells and the linkage between plasma membrane and metabolic activity, there is now evidence that K+ channels are involved in the regulation of cell proliferation [1]. The cellular mechanisms linking K+ channel activity and cell proliferation remain unclear, although one possibility is that activation of K+ channels might be required for the passage of cells through a specific phase of the mitotic cycle[1, 2]. Interestingly, recent studies revealed that HERG K+ channels are aberrantly expressed in some endometrial and colorectal cancers, and glioblastoma multiforme, but are absent from the normal cells from which the respective cancer cells are derived[3-8]. It has been proposed that the altered setting of the plasma membrane electric potential(Vrest) of cancer cells can contribute to tumor growth[9].

Our studies have shown that proliferation of tumor cells can often be modulated by the activity of K+ channels, encoded by the human ether a-gò-gò related gene(herg)[7]. In leukemia[8, 10] and neuroblastoma cells[6, 11], HERG channel activity modulates the progression through the mitotic cycle. In these cells an alterna-

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tive transcript of the herg1 gene was cloned and shown to be mainly expressed during the S phase of the cell cycle[6]. On the whole, data gathered thus far suggest that HERG channels may play a prominent role in the control of tumor cell proliferation[6, 8, 10] and apoptosis[12]. Our study has shown that the HERG protein encoded by the herg1 gene is overexpressed in gastric cancer and modulates the tumor cell proliferation and apoptosis. Thus, the HERG protein could be a molecular predictive indicator in gastric cancer. Based on this, we studied the expres-sion of HERG channels and their role in gastric cancer tumor progression.

MATERIALS AND METHODS

Cell culture and tissue samples

SGC-7901 and AGS(The China Center for Type Culture Collection, Wuhan, China) were cultured in RPMI 1640 medium(Hyclone, Logan, UT, USA) containing 10% FCS(Gibco, Invitrogen, Shanghai, China) and antibiotics(penicillin 100 units/ml and streptomycin 100 μg /ml, Hyclone) and incubated at 37℃ in a humidified atmosphere with 5% CO_2.

Tissue specimens obtained from neoplastic and normal nearby mucosa(collected at least 10 cm from the primary tumor), were obtained at surgery from patients suffering from gastric cancers, after written informed consent was obtained. Patients were all treated at the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Patients affected by viral hepatitis or who had undergone prior combined neoadjuvant radiochemotherapy were excluded from the study. Clinicopathological characteristics of patients enrolled in the study(evaluated by two different pathologists) were reported in ***Table 1***. Tissue samples were taken in the operating room and immediately frozen in liquid nitrogen, or fixed in 4% formaldehyde in PBS prior to being embedded in paraffin. Particular care was taken in sampling normal mucosa to avoid the presence of muscularis propria, which had been demonstrated to express the herg1 gene and HERG1 protein[13].

Table 1 **Clinicopathological characteristics of patients**

	No. of patients	Percentage
Sex		
M	38	59%
F	26	41%
Age		
≤ 50	22	34%
> 50	42	66%
WHO histological type Papillary and tubular adenocarcinoma	46	72%
Mucoid adenocarcinoma and ring-cell carcinoma	10	16%
Squamous cell and undifferentiated carcinoma	8	12%
Depth of invasion		
Mucosa	4	6%
Muscularis	15	24%
Serosa	45	70%
Differentiation		
Well differentiated	10	16%
Poorly differentiated	34	53%
Undifferentiated	20	31%
Lymph node metastasis		
Positive	46	72%
Negative	18	28%
Metastases		
Yes	10	16%
No	54	84%

Reverse transcription(RT) and polymerase chain reaction(PCR)

Reverse transcription-PCR for the herg1 and β-actin genes was performed as reported previously[7,8]. Harvested frozen cells were homogenized in a guanidinium thiocyanate solution, and total RNA was extracted, and the RNA purity and integrity was checked by running an aliquot on a 1.5% agarose gel. cDNA was then synthesized from 2 μg of RNA using 200U reverse transcriptase(Qiagen, Pudong, Shanghai, China) plus 200 μM each of dNTP and 2.5 μM random hexamers, in a 20 μl final reaction volume for 50 min at 42℃ and 15 min at 70℃. Two μl of cDNA were then amplified by polymerase chain reaction in a 50 μl reaction containing DNA polymerase 2.5 U, 200 μ M dNTPs, and 1x polymerase reaction buffer. The sequence of oligonucleotide primers(Invitrogen) was as follows: Primer sense:5′-TCCAGCGGCTGTACTCGGGC-3′;

Primer antisense:5′-TGGACCAGAAGTGGTCGGA-GAACTC-3′. These primers comprise a sequence between nucleotide 2171 to nucleotide 2746 of the human herg sequence, giving rise to a band 575 bp long. We carried out 43 cycles of amplification after 10 min of enzyme(Qiagen) activation at 94℃, denaturation at 94℃ for 1.5 min, anealing at 65℃ for 3 min, and extention at 72℃ for 1.5 min. DNA products were run on a 1.5% agarose gel(BD Pharmingen, USA) using a molecular weight marker, the 100bp DNA ladder (Invitrogen), and bands were visualized by ethidium bromide staining using an ultra-violet transilluminator. Control amplifications were performed by not adding DNA, RNA and retrotranscribed RNA in the PCR tube, but non-specific bands were observed. cDNA samples were checked for integrity by PCR detection of β-actin gene using the same conditions described above and using the primers giving rise to a band 247bp long as follows:Primer sense:5′- AACTCCATCATGAA-GTGTGA-3′; Primer antisense:5′- ACTCCTGCT-TGCTGATCCAC -3′.

Analysis of cell proliferation

SGC-7901 cells were seeded at a density of 1×10^6 in 1 ml of mixture containing RPMI 1640 and 10%FCS. Each was then seeded in triplicate wells of a 96-well microplate in the absence or in the presence of the specific HERG channel blocker, E-4031(Roche Pharmaceuticals, USA) at different concentrations(1 μ mol/L, 5 μ mol/L, 10 μmol/L, 20 μmol/L, and 40 μmol/L). The microplate was then incubated at 37℃ in a humidified atmosphere in 5% CO_2 in air for 48 hours. MTT 100 μL (5 g/L) was added to the cells and cultivated for another 4 hours. After the supernatant fluid was removed, DMSO(BD),100 μL per well, was added to the cells and shaken for 15 min. The absorbance at 490 nm was measured with an ELISA plate reader. At the same time, the cells without treatment served as controls. Each assay was repeated three times.

Cell cycle analysis

SGC-7901 cells were seeded at a density of 1×10^6 in a 1 ml of mixture containing RPMI 1640 and 10% FCS. Each was then seeded in triplicate wells of a 6-well microplate in the absence or presence of the specific HERG channel blocker, E-4031. The final blocker concentration was 40 μmol/L. The microplate was then incubated at 37℃ in a humidified atmosphere in 5% CO_2 in air for 48 hours. Cells were harvested, washed with PBS, fixed in 70% ethanol, then stained with 20 μg/ml propidium iodide containing 20 μg/ml RNase (DNase free, Qiagen) for 1 hour. The stained cells(1×10^6 cells/ml) were analysed by flow cytometry. The populations of G0/G1, S, and G2/M cells were quantitated using Cellquest Software. Results are expressed as the percentage of the cells in each phase.

Assay for apoptosis

SGC-7901 cells were seeded at a density of 1×10^5 in 1 ml mixture containing RPMI 1640 and 10%FCS. Each well was then seeded and incubated as described above in the cell cycle analysis section. Cells were harvested, and washed with PBS. FITC-conjugated Annexin V(Biovision, Shanghai, China), which binds to phosphatidylserine, and 7-aminoactinomycin(7-AAD, 20 μg/ml) were then added to the cells. After incubation for 15 min at room temperature in the dark, the cells were analyzed by flow cytometry. Early apoptotic cells were stained with Annexin V alone, whereas necrotic and late apoptotic cells were stained with both Annexin V and AAD.

Statistical analysis

Data were expressed as mean ± SD. Comparisons among groups were made by ANOVA(F-test), and a paired t-test was used for single comparison. A two-tailed $P < 0.05$ was taken to indicate a statistically significant difference between mean values.

RESULTS

Expression of the herg RNA

To study the presence of HERG in primary gastric cancer, we analyzed gastric cancer cells and gastric tissues, evaluating the expression of herg mRNA by RT-PCR, amplifying a 575 bp product and β-actin in gastric cancer cells. The fluorescence intensity of each ethidium bromide-labeled product was first normalized to the β-actin signal from the same sample. The herg mRNA was expressed in all of the 64 primary cancer cases but not in normal gastric tissues from those patients. The expression of herg mRNA did not associate with the clinical and cytogenetic features of the gastric cancers. HERG K+ cells from the human colon cancer cell line HT-29 and colorectal cancer tissue samples were then examined, and herg PCR bands were also found in these cells(***Fig. 1***).

Effects on proliferation of gastric cells by blocking HERG K+ channels

To investigate the effects of E-4031, a HERG K+ channel blocker, on the proliferation of SGC-7901 cells, cell proliferation was analyzed using the MTT assay. As shown in ***Fig. 2***, SGC-7901 cell proliferation was significantly inhibited by E-4031. However, the same inhibitor did not affect the proliferation of cells not expressing herg(data not shown). The inhibition of SGC-7901 cell proliferation by E-4031 was dose-dependent, reaching a maximum at 40 μmol/L. These results indicate that the HERG K+ channel was required

for gastric cancer cell proliferation.

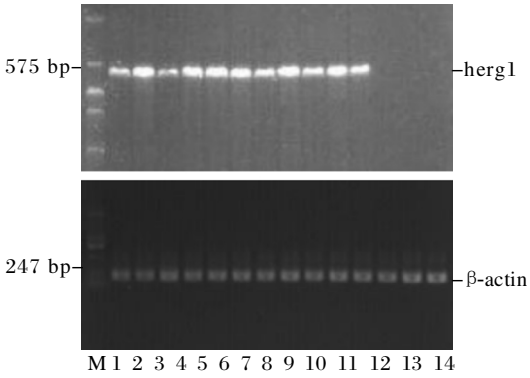


Fig. 1 herg RNA expression in normal and gastric cancer cells

Rt-PCR experiments were performed on normal and neoplastic gastric tissues, SGC-7901, and HT-29 cell lines. Lane1:Marker; Lane 2:SGC-7901 cells, Lane 3:HT-29 cells, Lanes 4-11:neoplastic gastric tissues, Lanes 12-13:normal gastric tissues, Lane14:negative control.

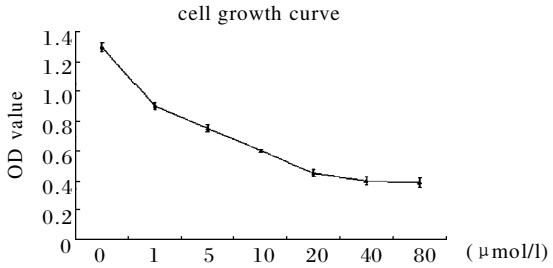


Fig. 2 Inhibitory effects of HERG K+ channel specific inhibitor, E-4031, on the proliferation of SGC-7901 cells after treatment for 48 hours

Proliferation was assessed using MTT assay. The cell proliferation was reduced by E-4031 and the inhibition was dose-dependent, reaching a maximum at 40 μmol/L.

To examine the effect of HERG K+ channel inhibitor on cell cycle modulation, SGC-7901 cells were not treated or treated with E-4031.The cells were then subjected to cell cycle analysis by flow cytometry. According to the flow cytometry profile shown in ***Fig. 3***, E-4031 could effectively alter cell cycle distribution of the growing SGC-7901 cells. After 48 hours of the treatment, this channel blocker caused a marked reduction of the cell population in the S phase, along with a significant increase in the number of cells in G1 phase. Similar results were also observed using AGS cells,(data not show). For instance, the G1/G0 ratio increased from 33.04% to 45.83% in the treated cells. These results clearly suggest that E-4031 suppresses the cell growth by inducing a specific block at the G1/S transition phase of the cell cycle. However, as evidence from ***Fig. 3***, the progression through G2/M phase was not affected by the HERG K+ channel inhibitor.

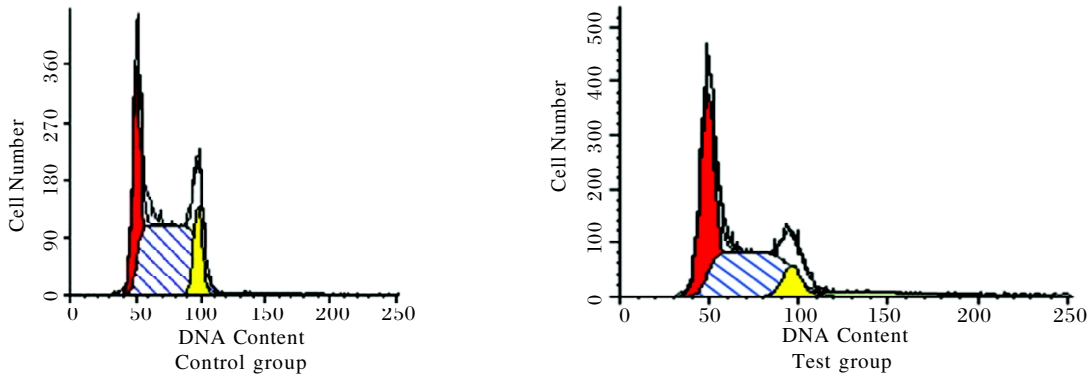


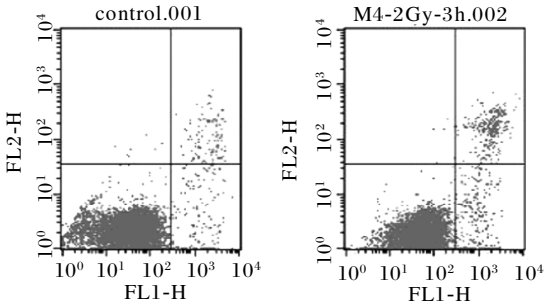
Fig. 3 Cell cycle distribution of SGC-7901 cells following treatment of E-4031, a HERG K+ channel-specific blocker

Synchronized cells at the G1/S boundary were incubated with E-4031 and immmediately released to permit resumption of cycling for 48 hours before being subjected to flow cytometric analysis. Control cells in G0/G1, S and G2/M stages of the cell cycle were 33.04% ± 1.15, 58.96% ± 0.52, and 8.0% ± 0.79 respectively, whereas E-4031-treated cells in these stages were 45.83% ± 2.68, 46.17% ± 3.5, and 8.0 ± 0.86. The HERG blocker E-4031 increased the cell population in the G0/G1 phase($P < 0.05$). (The first peak of the curve in red represents the G0/G1 phase, and the second peak of the curve in yellow represents the G2/M phase, and the section of the curve between the two peaks in white color represents the S phase).

Effects of blocking HERG K+ channels on the apoptosis of gastric cells

To explore whether HERG K+ channels activity is associated with apoptosis in gastric cancer cells, we studied the effect of HERG K+ channel blockade on SGC-7901 cells. As shown in ***Fig. 4***, E-4031 caused a significant increase in the number of cells in the right quadrants that represent the apoptotic and late apoptotic/necrotic cells, and a significant decrease in the percentage of viable cells(lower left quadrant).

The cells were incubated for 48h(1×10^4cells/ml) in the absence(Control group) or presence(Test group) of 40 μmol/L E-4031. The dead cells(apoptosis/necrosis) were visualized by Annexin V binding/AAD staining.



Quad	% Gated
UL	0.13
UR	1.96
LL	95.13
LR	2.79

Control group

Quad	% Gated
UL	0.09
UR	5.04
LL	90.43
LR	4.44

Test group

Fig. 4 Effect of E-4031 on cell apoptosis in SGC-7901 cells

For this apoptosis analysis, the numbers in each quadrant indicate the percentage of cells from a total of 10,000 cells. Viable cells appear in the lower left-hand quadrant, whereas cells in the right-hand quadrants denote apoptotic(lower quadrant) and apoptotic/necrotic(upper quadrant) cells. The viable, apoptotic, and apoptotic/necrotic cells in the control group were respectively 95.13% ± 2.75, 2.79 ± 0.45%, and 1.96 ± 0.36 %, whereas E-4031-treated cells in three quadrants were respectively 90.43% ± 2.68, 4.44% ± 0.72, and 5.04 ± 0.86. The change in the apoptotic cell population was statistically significant($P < 0.05$).

DISCUSSION

Evidence has been accumulating from laboratory studies indicating that tumor cells possess various types of K+ channels, and their pathophysiologic functions have also attracted great attention. Results reported in this study are the first to demonstrate that herg is expressed in both gastric cancer tissues and cell lines.

HERG K+ channels have been reported to show differential expression patterns in tumor cell lines. Overexpression of HERG has been suggested to represent a selective advantage for these tumor cells[3-8]. In this paper we provided evidence that herg mRNA was expressed in all the gastric cancer tissues examined, but not in normal tissues. In the gastric tumor tissue, the expression of herg mRNA did not correlate with the clinical and cytogenetic features of the tumors. HERG K+ channels were also expressed in SGC-7901 and AGS cells, as implied by the effects of a specific channel blocker on these cells. The results of this study, together with the other findings mentioned above, indicated that K+ channels belonging to the HERG family may have oncogenic potential.

Pharmacological evidence indicated that K+ channels might be involved in the cell cycle and proliferation. A well-documented case of the role of K+channels in proliferation and differentiation stemmed from lymphocytes[14]. Our results also showed that specifically blocking the HERG K+ channel could reduce gastric cancer cell proliferation, while it did not affect the growth of the cells not expressing these channels. HERG K+ channels could be acting as a regulator of gastric cancer cell proliferation, because when these K+ channel sare inhibited, proliferation was impaired. This suggested that gastric cancer cells need HERG K+ channel to proceed with the cell cycle.

According to the flow cytometry profile, shown in ***Fig. 3***, E-4031 could effectively alter cell cycle distribution of the growing SGC-7901 cells. After 48 hours of the treatment, this agent caused a marked reduction of the percentage of cells in the S phase, along with a significant increase in cell numbers in the G1 phase. Similar results were also observed using the AGS cells (gastric carcinoma cell line, data not show). For instance, the G1/G0 ratio increased from 33.04% to 45.83% in the treated cells. These results clearly suggested that E-4031 suppresses the cell growth by inducing a specific block at G1/S transition of the cell cycle. However, it was evident that the progression through G2/M phase was not affected by the HERG K+ channel inhibitor.

The data presented in this report demonstrate that HERG K+ channel affected cellular proliferation, in part by increasing the cells in the S phase, although further research is required to explore the molecular basis of this event. The present finding demonstrated that blockage of HERG K+ channels by E-4031 induces growth arrest of gastric cancer cells in the G1 phase. HERG K+ channel activity was therefore necessary for the proliferation of gastric cancer cells.

Our result also showed a direct effect of E-4031 on SGC-7901 cell apoptosis. As shown in ***Fig. 4***, not only the apoptotic cells but also the necrotic cells were increased by blockade of the HERG K+ channels. A significant change in the apoptotic cell population was seen ($P < 0.05$). These data indicated that HERG K+ channels protect tumor cells, such as leukemia cells and SGC-7901 cells, from apoptosis, inhibiting cell death and facilitating cell survival.

Our findings may have clinical importance. As a crucial cellular function, cell proliferation is very strictly controlled by several independent mechanisms. One major characteristic of gastric cancer cell biology is the failure of the control of cell growth. Tumor cells proliferation can be virtually unlimited. Hence, developing a method of inhibiting the genesis of tumor cell proliferation is a promising avenue for therapy. HERG K+ channels clearly favor tumor cell proliferation. Therefore, inhibition of HERG K+ channel function or

down-regulation of HERG K+ channel expression should inhibit tumor genesis. Thus, HERG K+ channels can be considered as a potential pharmacological target for cancer therapy. On the other hand, our increasing understanding of cancers has allowed a reassessment of our clinical approach to gastric cancer. With regard to the current therapy, the prognosis for gastric cancer is poor. Many drugs can usually ablate gastric blast cells but may not effectively target the cancer population. Herg is expressed in gastric cancer cells but not expressed in normal cells. The use of K+ channels for therapy of autoimmune diseases has become an accepted methodology[15]. Thus, the concept of identifying and targeting K+ channels found on the surface of cancer appears to be a very useful direction for future research.

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