Alcohol Consumption Promotes the Invasion and Proliferation of Gastric Cancer

Jie Ding[#], Hao Wang[#], Xing Kang, Feng Wang, Hong Chen, Min Feng, Wen Xian Guan

Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China

Abstract: Objective To determine the effects of alcohol on the invasion and proliferation of gastric cancer. **Methods** In an *in vitro* study, gastric cancer cells were treated with different concentrations of alcohol. The protein levels of matrix metalloproteinase (MMP)2/7/9/13, Cyclin D and PCNA were determined by Western blot. The transcription levels of MMP2/7/9/13 were determined by qRT-PCR. An MMPs inhibitor (GM6001) was used to analyze the role of MMPs in the invasion of gastric cancer cells. In an *in vivo* experiment, the effects of alcohol on the proliferation of gastric cancer was analyzed in a heterotopic transplantation animal tumor model. **Results** The protein levels of MMP2/7/9/13 were increased in gastric cancer cells (AGS and HGC-27) compared with normal gastric epithelial cells (GES). Alcohol treatment resulted in further elevation of the MMPs expression in the cancer cells and promoted the invasion of the gastric cancer cells. GM6001 inhibited these effects. In addition, alcohol treatment promoted the expression of Cyclin D and PCNA in cancer cells and gastric tumor proliferation in the animal model. **Conclusion** Alcohol consumption promoted the invasion and proliferation of gastric cancer. Confirmation should be obtained in further studies in additional models.

Key words: Gastric cancer; Alcohol; Invasion; Proliferation; Matrix metalloproteinases

Introduction

Cancer is a leading cause of human death, and produces serious economic burdens. An epidemiological study published in 2018 provided a status report on the global burden of 36 cancers in 185 countries. The incidence of gastric cancer ranked fifth in the world, and was third for cancer-related mortality [1]. In China, gastric cancer is the second most common cancer and the third leading cause of death from malignant disease [2].

Alcohol is one of the most commonly and regularly used chemical substances. The stomach is one of the major target organs of alcohol. Epidemiological studies indicated that alcohol increases both the incidence and mortality of gastric cancer [3,4]. However, the exact effects of alcohol on gastric cancer and the underlying mechanism (s) by which alcohol promotes gastric cancer development are still unclear.

Degradation of the extracellular matrix (ECM) is an important step in tumor metastasis [5]. Extracellular matrix-degrading enzymes, such as matrix metalloproteinases

(MMPs), play an important role in the pathological and physiological processes of cancer development [6]. MMPs and their tissue inhibitors (TIMPs) were found to be associated with gastric cancer development. For example, MMP2 is implicated in the effects of p21-activated protein kinase 1 on the invasion of gastric cancer cells [7], while isocitrate dehydrogenase 2 inhibited gastric cancer cell invasion by decreasing the level of MMP7 [8]. Clinical studies showed that MMP9/13 play critical roles in the incidence and prognosis of gastric cancer [9,10]. In addition, MMP1/3/10/12/14 and TIMP1/2 were all reported to be associated with the development of gastric cancer [11-17].

Proliferation is another important biological feature associated with the prognosis of cancer. Alcohol was found to promote colorectal cancer proliferation by leading to hypermethylation of ADHFE1 [18]. However, the role of alcohol in gastric cancer proliferation remains unclear [19]. Our previous studies indicated that alcohol consumption might result in endoplasmic reticulum stress (ER-stress) [20]. The ER is the site of protein folding, modification, assembly and intracellular calcium storage. An ER-stress-related inflammatory response can occur via nuclear factor-kappa B (NF-κB) and C-jun amino terminal kinase (JNK)/transcription factor activator protein 1 (AP-1) [21,22]. Chronic inflammation is associated with the occurrence, proliferation and metastasis of tumors, suggesting that alcohol-induced ER stress may lead to inflammation and the subsequent proliferation and metastasis of cancer cells [23].

In this study, we investigated the effects of alcohol on the invasion and proliferation of gastric cancer.

[#]Jie Ding and Hao Wang made an equal contribution to this paper.
Corresponding author: Wen Xian Guan, MD, PhD, Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China; Tel: +86 25 6818 2099; Fax: +86 25 6818 2097 or +86 158 5050 2391; Email: guan-wx@163.com; Min Feng, MD, PhD, Department of General Surgery, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, Jiangsu 210008, China; Tel: +86 25 6818 2099; Fax: +86 25 6818 2097; Email: fengmindocor@163.com

Materials and Methods

Cell culture

Human gastric cancer cell lines (AGS and HGC-27) and human gastric mucosal endothelial cells (GES-1) were purchased from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco, Gaithersburg, MD, USA) at 37°C in a humidified atmosphere with 5% CO₂. Cell lines were cultured with ethanol at 0 mmol/L, 35 mmol/L, or 85 mmol/L in serum-containing culture medium for 24h.

Invasion and cell migration assay

The effects of alcohol on the invasion of cancer cells was determined using a modified Boyden chamber invasion assay [24]. Cells were cultured to 80% confluency and serum-starved overnight. The cells were then trypsinized and a viable cell count was obtained. Homogeneous single cell suspensions (1×10^5 cells/well) were added to the upper chambers in serum-free medium containing alcohol (0/35/85 mmol/L) and/or GM6001 (GM, 0.2 nmol/L, MCE, Monmouth Junction, NJ, USA). An equal volume of the same medium containing 10% FBS was placed in the lower chamber (the well beneath the filter) to act as a chemoattractant. The assay plates were incubated at 37°C for 24h. Following incubation, the filters were fixed with 3% glutaraldehyde in phosphate-buffered saline and stained with crystal violet for cell quantification.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from each cell line with the RNAPure tissue kit (Thermo Fisher Scientific, Inc. Waltham, MA, USA) following the manufacturer's instructions. The expression levels of mRNA for MMP2/7/9/13 and GAPDH were determined by qRT-PCR with an ABI Prism 7900HT (Applied Biosystems). Values were calculated as n-fold differences in gene expression relative to GAPDH. The following sets of primers were used in the PCR amplification: GAPDH (forward, 5' GAA GGT GAA GGT CGG AGT 3' and reverse, 5' GAA GAT GGT GAT GGG ATT TC 3'); MMP2 (forward, 5' GAG AAC CAA AGT CTG AAG AG 3' and reverse, 5' GGA GTG AGA ATG CTG ATT AG 3'); MMP7 (forward, 5' GAGTGAGCTACAGTGGGAACA 3' and reverse, 5' CTATGACGCGGGAGTTTAACAT 3'), MMP9 (forward, 5' GGG AGA CGC CCA TTT CG 3' and reverse, 5' CGC GCC ATC TGC GTT T 3'), MMP13 (forward, 5' CAG ACT TCA CGA TGG CAT TGC 3' and reverse, 5' AGG AAAAGC ATG AGC CAG CA 3').

Western blot analysis

Whole cell lysates were prepared as described

previously [20]. Total protein concentrations were determined using a bicinchoninic acid protein assay kit (KeyGen, Jiangsu, China). Equal amounts (30 µg) of protein extracted from the cultured cells were run on 10% SDS-PAGE, followed by electro-transfer to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk powder for 2h. Then, the membranes were incubated overnight at 4°C with the primary antibodies, anti-GAPDH, anti-Cyclin D, anti-PCNA, anti-MMP2, anti-MMP7, anti-MMP9 and anti-MMP13 (all from Abcam, USA), then with horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (ZSGB-Bio, Beijing, China) for 2h at room temperature. The membranes were visualized by chemiluminescence (Millipore) and exposed with an automatic exposure machine (Bio-Rad, USA) in a dark room, and then Quantity One software was used for gray-scale analysis.

Animal model

Male BALB/C nude mice (18-20g) were obtained from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animals were treated according to the guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Research Advisory Committee at Nanjing University (Nanjing, China). Briefly, HGC-27 cells (5×10^5 cells/mouse) were subcutaneously transplanted into the axillary area of nude mice. The mice were housed in a SPF barrier environment after the completion of feeding. Tumor nodules (0.3 × 0.3 cm) were observable in all of the mice with naked eye after a week. At that time, the mice started to receive water containing ethanol (85 mmol/L) by oral gavage, which continued for three weeks. Thereafter, the tumor-bearing mice were sacrificed, tumor tissues were isolated, and the weights and volumes of the tumors were determined. The tumor volume was measured by a Vernier caliper and calculated as $[\text{length} \times (\text{width})^2]/2$.

Statistical analysis

Data are presented as the means ± SD. For the statistical analysis of differences between two groups, Student's t-test for independent means was used. A value of $P < 0.05$ was considered to be statistically significant, and a P -value < 0.01 was considered to be very significant. The statistical analysis was performed using the SPSS 19.0 software.

Results

The baseline expression of MMPs was elevated in gastric cancer cells

We examined the MMP2/7/9/13 mRNA and protein expression levels in two cancer lines (AGS and HGC-27) and non-malignant gastric mucosal cells (used as a control; GES-1). It was found that MMP2 was more highly expressed in HGC-27 cells, while the MMP7/9/13 protein

levels showed no significant differences compared with the GES-1 cells (Figure 1A and B). In AGS cells, the protein level of MMP7, but not MMP2/9/13, was significantly elevated compared with the GES-1 cells (Figure 1A and B). The transcription levels of MMPs were almost consistent with protein levels, but the MMP2 mRNA level was significantly increased in HGC-27 cells, but not AGS cells (Figure 1C).

Alcohol treatment promoted MMPs expression in gastric cancer cells

To determine the effects of alcohol on MMPs expression in gastric cancer cells, we treated the AGS and HGC-27 cells with different concentrations of alcohol and then determined the MMP2/7/9/13 protein and mRNA levels. We found that the protein levels of MMP2/7 were increased in AGS cells after alcohol treatment (35 mM and 85 mM), while the protein levels of MMP9/13 were significantly increased only when exposed to the higher concentration of alcohol (85 mM). In HGC-27 cells, the MMP7/9/13 protein levels were increased after exposure to both concentrations of alcohol. The MMP2 protein level was increased only after treatment with the higher concentration of alcohol (Figure 2A and B). With regard to the transcriptional levels, the MMP2/7/9 expression levels were increased in AGC cells after exposure to both concentrations of alcohol, while the MMP13 mRNA level was increased only after treatment

with the higher concentration of alcohol. The mRNA levels of MMP2/7/9/13 in the HGC-27 cells were increased by both concentrations of alcohol (Figure 2C).

Alcohol treatment promoted the invasion of gastric cancer cells via MMPs

MMPs play a critical role in cancer cell invasion [7,8]. To evaluate the effects of alcohol on gastric cancer cell invasion, we selected HGC-27 cells for an invasion experiment given their stronger response to alcohol stimulation noted above. We found that alcohol treatment promoted gastric cancer cell invasion in a concentration-dependent manner (Figure 3). To clarify the role of MMPs in the effects of alcohol on gastric cancer cell invasion, GM6001 was used to neutralize MMPs. GM6001 is a broad-spectrum MMPs inhibitor that inhibits the activity of MMPs-1/2/3/7/8/9/12/14/26 [25]. We found that GM6001 suppressed the invasion of cancer cells and prevented the stimulatory effects of alcohol (Figure 3).

Ethanol exposure stimulated the proliferation of gastric cancer cells

Proliferation is an important biological feature associated with cancer development [26]. We found that the protein levels of Cyclin D and PCNA, two markers of cell proliferation [11,12], were increased in HGC-27 cells after alcohol treatment (35 mM and 85 mM), while in the AGS

Figure 1 The expression of MMPs is elevated in gastric cancer cells. (A and B) The protein expression levels of MMP2, MMP7, MMP9 and MMP13 in GES-1, AGS and HGC-27 cells were determined by Western blot. (C) The mRNA levels of MMP2, MMP7, MMP9 and MMP13 in GES-1, AGS and HGC-27 cells were determined by qRT-PCR. The results were normalized to GAPDH. Data were expressed as the means $\pm$ SD. $n=5$, $*P < 0.05$.

cell line, alcohol stimulation only resulted in a significant increase in the Cyclin D protein level (Figure 2D).

Alcohol consumption promoted the growth of heterotopic gastric cancer tumors in mice

To further understand the effects of alcohol on gastric cancer proliferation, we carried out an *in vivo* study. In the *in vivo* study, HGC-27 cells were used to establish an animal heterotopic tumor transplantation model as described in the Materials and Methods section. Alcohol treatment (via oral gavage to simulate normal consumption) promoted gastric

cancer tumor growth, with increased tumor volumes and weights compared to the controls administered water (Figure 4).

Discussion

Alcohol use or abuse is a leading risk factor for various diseases. A recent study indicated that the global adult alcohol consumption per-capita increased from 5.9 L in 1990 to 6.5 L in 2017, and would increase to 7.6 L by 2030 [27]. Epidemiological studies have found that alcohol was associated with the occurrence and prognosis of gastric

Figure 2 Alcohol promotes MMPs, Cyclin D and PCNA expression. (A and B) AGS and HGC-27 cells were treated with different concentrations of alcohol, then the protein expression levels of MMP2, MMP7, MMP9 and MMP13 were determined by Western blot. (C) The mRNA levels of MMP2, MMP7, MMP9 and MMP13 were determined by qRT-PCR. The results were normalized to GAPDH. Data were expressed as the means $\pm$ SD. $n = 5$, * $P < 0.05$. A1, alcohol treatment (35 mM); A2, alcohol treatment (85 mM).

Figure 3 Alcohol promotes the invasion of gastric cancer cells. HGC-27 cells were plated into an invasion assay system as described in the Materials and Methods. After a 24h incubation, the filters were stained with crystal violet for quantification of the cells. Data were expressed as the means $\pm$ SD. $n = 3$, * $P < 0.05$.

Figure 4 Alcohol consumption promotes gastric cancer tumor growth. HGC-27 cells were used to establish a heterotopic transplantation animal tumor model. The animals were sacrificed after 3 weeks of alcohol treatment (85 mM). The volume and weight of tumors were measured and compared with controls. Data were expressed as the means $\pm$ SD. $n = 3$, * $P < 0.05$.

cancer [3,4], while the underlying mechanism by which alcohol promotes gastric cancer development remains unclear. In this study, we investigated the role of alcohol in the invasion and proliferation of gastric cancer.

MMPs degrade the proteins in the ECM and increase tumor invasion and metastasis. MMP2/7 were reported to play a critical role in gastric cancer invasion [7,8], while MMP9/13 were found to be related to the prognosis of gastric cancer [9,10]. Our previous study demonstrated that MMP9/13 were associated with colon cancer metastasis [28]. In this study, we found that the baseline MMP2 expression was increased in HGC-27 cells and the MMP7 level was increased in AGS cells, although MMP9/13 did not show any significant elevation in either of the cancer cell lines compared with GES-1 cells.

Alcohol consumption was reported to increase the occurrence of gastric cancer and lead to a worse prognosis in clinical studies [3]. In this study, we found that alcohol treatment promoted MMP2/7/9/13 expression in both AGS and HGC-27 cells. However, the degree of MMPs elevation was different in the two cell lines. The response of HGC-27 cells to alcohol stimulation in MMPs expression was stronger, so we selected these cells for the invasion experiment. We found that alcohol promoted the invasion of gastric cancer cells in a concentration-dependent manner.

In this study, we used GM6001 to confirm the role of MMPs in gastric cancer cell invasion. It was found that GM6001 significantly suppressed HGC-27 invasion and decreased the effects of alcohol. These data indicate that alcohol exposure promotes gastric cancer invasion by increasing MMPs expression.

Proliferation is associated with tumor development and prognosis [26]. In this study, we found that the Cyclin D level was increased in both HGC-27 and AGS cells after alcohol treatment. The PCNA level was also increased, but only in HGC-27 cells. We used the HGC-27 cell line to establish a heterotopic transplantation animal tumor model. It was found that alcohol consumption promoted gastric cancer tumor growth, with increased tumor volume and weight.

Conclusion

In conclusion, this study demonstrated that alcohol promotes gastric cancer invasion and proliferation. The elevation of MMPs after alcohol treatment contributed to the invasion of the cancer cells. Although the underlying mechanism(s) by which alcohol consumption promotes gastric cancer proliferation remain unclear, this study provides information on how ethanol may lead to gastric cancer development and progression, and provides a stepping stone for further studies.

Conflict of Interests

The authors of this manuscript declare no conflicts of interest.

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