Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (1): 1-5
Published online March 5, 2014 (http://www.cellmolbiol.com)
Received on November 28, 2013, Accepted on February 17, 2014.
doi : 10.14715/cmb/2013.60.1.1

RNAi-mediated silencing of ATP-binding cassette C4 protein inhibits cell growth in MGC80-3 gastric cancer cell lines

L. Chen[1], J. Gu[1], L. Xu[2], C. Qu[2], Y. Zhang[2] and W. Zhang[1]

[1] Department of Surgery, Xinhua Hospital, Medical College of Shanghai Jiaotong University, Shanghai 200092, China
[2] Endoscopy Center, Xinhua Hospital, Medical College of Shanghai Jiaotong University, Shanghai 200092, China

Corresponding author: Wenjie Zhang, Department of Surgery, Xinhua Hospital, Medical College of Shanghai Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, China. Tel.: +86-21-25078999, Fax: +86-21-65153984, E-mail: wenjiezhangdr@163.com

Abstract

ATP-binding cassette C4/multidrug resistance associated protein 4 (ABCC4/MRP4) is a relatively well-studied member of the ATP-binding cassette drug transporter family. Recent studies suggested that ABCC4 is implicated in the development of several human cancers. In the present study, we report that knockdown of ABCC4 by lentivirus-delivered short hairpin RNA could inhibit gastric cancer cell growth in vitro. The proliferation of MGC80-3 cells was significantly reduced after infection with Lv-shABCC4, as determined by MTT and colony formation assays. In addition, knockdown of ABCC4 blocked the cell cycle progression in MGC80-3 cells. To our knowledge, it is the first report to investigate the role of ABCC4 in gastric tumorigenesis. Our data provides additional evidence that ABCC4/MRP4 could be a potential therapeutic target for gastric cancers.

Key words: ABCC4, gastric cancer, shRNA, proliferation.

Introduction

Gastric cancer is one of the major malignancies in mainland of China, with high incidence and mortality (1). Newly diagnosed gastric cancer patients in China account for about 40% worldwide cases every year (2). Over 80% of gastric cancer diagnoses are at an advanced stage, resulting in relatively poor outcomes (3, 4). Despite intensive research and major advances over the past few decades, gastric cancer therapy remains an intractable challenge (5). Development of novel therapeutics to target molecules crucially involved in gastric cancer growth is urgently needed.

ATP-binding cassette C4/multidrug resistance associated protein 4 (ABCC4/MRP4) is a relatively well-studied member of the ATP-binding cassette (ABC) drug transporter family, which has broad substrate specificity that facilitates the transport of bile salts and conjugates nucleoside analogs and drugs (6-8). Recent studies suggested ABCC4 as a key player in the development of several human cancers (9-11). For instance, ABCC4 protein was found to be upregulated in a great number of pancreatic cancer specimens, and further in vitro studies showed that downregulation of ABCC4 expression in pancreatic cells significantly inhibited their proliferation and colony formation (10). In human leukemia, ABCC4 is implicated in the control of cell proliferation and differentiation by regulating cellular cAMP levels (11). Moreover, ABCC4 is associated with complete remission rate in patients with acute myeloid leukemia (AML) in first relapse treated with fixed-dose-rate gemcitabine and mitoxantrone (12). In a previous study, the expression of ABCC4 is found to be elevated in human colorectal cancer specimens (in comparison to normal mucosa) and in colorectal cancer cell lines (9). However, the regulatory mechanism of ABCC4 in human cancers remains to be exploited.

In this study, in order to investigate the role of ABCC4 in gastric cancer, we employed lentivirus-mediated short hairpin RNA (shRNA) to silence ABCC4 expression in human gastric cancer cell line MGC80-3. We found that knockdown of ABCC4 could markedly decrease cell proliferation and colony formation. In addition, cell cycle progression was also dampened after infection with Lv-shABCC4. Hence, we conclude that ABCC4 may play a prominent role in gastric cancer growth in vitro.

Materials and methods

Cell culture

Human gastric cancer cell line MGC80-3 was obtained from the Cell Bank of Chinese Academy of Sciences (#TCHu 84, Shanghai, China). Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI1640) (Gibco, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in humidified atmosphere of 5% CO2.

RNA extraction and Quantitative real-time PCR (qRT-PCR)

Total RNAs of cultured cells were extracted respectively using the Trizol solution (Invitrogen, CA, USA) at 24 h after treatment. The cDNA was immediately reverse transcribed from isolated RNA with SuperScript III First-Strand Synthesis System (Invitrogen, CA, USA). ABCC4 mRNA expression was evaluated by real-time PCR using the SYBR Premix Ex TaqTM Perfect Real Time (TaKaRa, Shiga, Japan) on an ABIPRISM 7500 Real-Time System. β-actin was applied as the input reference. The primers used are as follows: β-actin, 5'-GGCGGCACCACCATGTACCCT-3' (forward) and 5'-AGGGGCCGGACTCGTCATACT-3' (reverse);

ABCC4, 5'-GTTCTTCTGGTGGCTCAATCC-3' (forward) and 5'-GGCTTCTGTGCGTCATTCTC-3' (reverse). Relative mRNA was determined using the formula 2-ΔCT (CT; cycle threshold) where ΔCT =CT (target gene) - CT (β-actin).

Construction of ABCC4 shRNA-expressing lentivirus

To construct shABCC4 stably expressing cell line, ABCC4 small interference RNA (siRNA) (5'-GCACTCATTAAATCACAAGAA-3') was inserted into the pFH-L plasmid (Hollybio, Shanghai, China). The non-silencing siRNA (5'- TTCTCCGAACGTGTCACGT-3') was used as control. The lentivirus-based shRNA-expressing vectors were constructed, confirmed by DNA sequencing and named as pFH-L-shABCC4 or pFH-L-shCon. For the transfection, MGC-803 cells (5×104) were seeded in 6-well plates and cultured for 72 h to reach 90% confluence, respectively. At 2 h before transfection, the medium was replaced with serum-free RPMI1640. The plasmid mixture containing pFH-L-shABCC4 (or pFH-L-shCon) and pVSVG-I pCMV△R8.92 packaging vectors, as well as Lipofectamine 2000 (Invitrogen, CA, USA) were added to the cells. At 5 h after incubation, the medium was replaced with RPMI1640 containing 10% FBS. Lentiviral particles (Lv-shABCC4 or Lv-shCon) were harvested at 48 h after transfection and purified by ultra-centrifugation. As the lentivirus carries green fluorescence protein (GFP), the viral titer was determined by counting GFP-expressing cells under fluorescence microscopy 96 h after infection.

Western blot analysis

Samples were homogenized in western blot analysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 0.28 kU/L aprotinin, 50 mg/L leupeptin, 1 mM benzamidine, and 7 mg/L pepstain A (all chemicals from Sigma-Aldrich, MO, USA). The homogenate was then centrifuged at 12,000 g for 15 min at 4°C and the supernatant was retained and preserved at −80°C for later use. Protein concentration was determined using a BCA kit (Pierce). Each sample was subject to electrophoresis on 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes on a semidry electrotransferring unit (Bio-Rad, CA, USA), and incubated with antibodies against ABCC4 (Santa Cruz Biotechnology, CA, USA), in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk overnight at 4°C. After the overnight incubation with the primary antibodies, membranes were washed and incubated with horseradish peroxidase-conjugated second antibody in TBST for 3 h. Immunoreactivity was detected with enhanced chemoluminescent autoradiography (ECL kit, Amersham Life Science, Buckinghamshire, UK). The membranes were reprobed with GAPDH (Santa Cruz Biotechnology, CA, USA) after striping.

MTT assay

Cell proliferation was determined using a colorimetric assay with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. After treatment with or without the appropriate plasmid, MGC80-3 cells were seeded in 96-well plates (1 × 104 cells/well). Cells were allowed to attach overnight and cell proliferation was evaluated for up to 5 days by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 10 µl of MTT reagent (5 mg/ml in PBS) was added to the cells and incubated for 4 h at 37°C. The medium was removed and 200 µl of isopropanol were added. The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 595 nm using an ELISA plate reader.

Plate colony formation assay

In vitro tumorigenicity was determined on the basis of cell growth in a plate colony assay. Cells (1 × 103 cells/well) were seeded in 6-well plates in RPMI1640 medium and cultured at 37°C in 5% CO2 for 10 days. Visible colonies were photographed by Giemsa staining (Merck, Darmstadt, Germany). Colonies with more than 50 cells were counted by Colony Counter software.

Fluorescence-activated cell sorting (FACS) analysis

The DNA contents of cell cycle phases can be reflected by varying propidium iodide (PI) fluorescent intensities. Therefore, cell cycle distribution of Lv-shABCC4- or Lv-shCon-infected cells was analyzed by flow cytometry assay following PI staining. In brief, cells were collected 96 h after infection with lentivirus containing Lv-shABCC4 and seeded in six-well plates (2×105 cells/well). Cells were allowed to attach overnight and collected. After washing with ice-cold phosphate buffered saline, cells were suspended in about 0.5 ml of 70% cold alcohol and kept at 4°C for 30 min. The cells were then treated with 100 mg/ml of DNase-free RNase and incubated for 30 min at 37°C. PI (50 mg/ml) (Sigma-Aldrich, MO, USA) was added directly to the cell suspension. The suspension was filtered through a 50-mm nylon mesh, and total of 10000 stained cells were analyzed by a flow cytometer (FACS Cali-bur, BD Biosciences, CA, USA).

Statistical analysis

Data were analyzed using GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software, CA, USA). Average values were expressed as mean ± SD of three independent experiments in triplicate. Statistical significance between groups was determined using Student's test. A p value less than 0.05 was considered statistically significant.

Results

Expression of ABCC4 was suppressed by infection with Lv-shABCC4 in gastric cancer cells

To suppress the expression of ABCC4 in gastric cancer cells, lentivirus that stably expressed ABCC4-specific siRNA (Lv-shABCC4) was constructed and MGC80-3 cells were infected (multiplicity of infection: 20). As shown in Figure 1A, infection efficiency is approximately 90% in both Lv-shCon-infected and Lv-shABCC4-infected MGC80-3 cells. Furthermore, the-

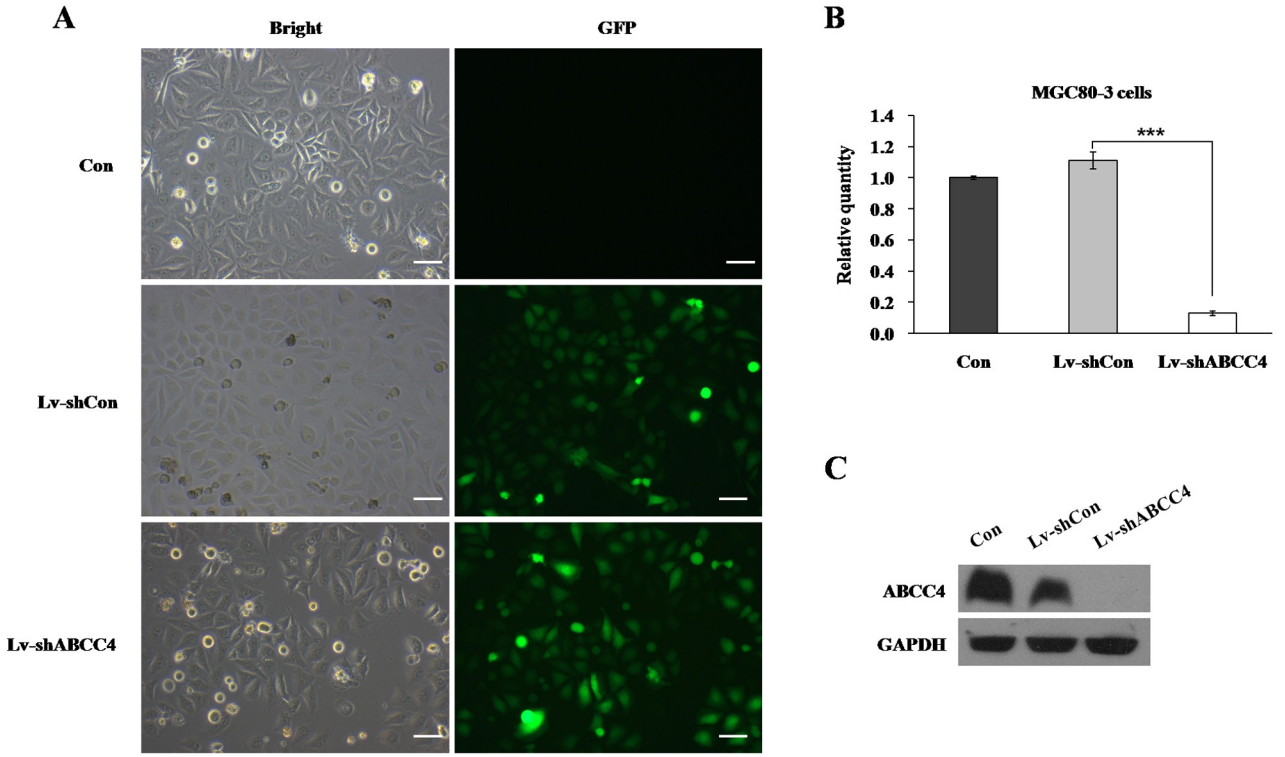

Figure 1. Expression of ABCC4 was suppressed by infection with Lv-shABCC4. (A) GFP expression in transfected cells was observed under light a microscope and a fluorescence microscope. Scale bar: 100 μm. (B) Expression analysis of ABCC4 mRNA in MGC80-3 cells after infection with lentivirus by qRT-PCR. β-actin was used as an internal gene. Data represent the mean ± SD of three independent experiments. ***p<0.001, compared with Lv-shCon. (C) The protein level of ABCC4 in MGC80-3 cells after infection with lentivirus was determined by western blot ana-

expression of ABCC4 mRNA was accordingly reduced in Lv-shABCC4-infected cells, compared with non-infected or Lv-shCon-infected cells (***p<0.001, Figure 1B). The protein level of ABCC4 in Lv-shABCC4-infected cells was also markedly lower than that in control cells (Figure 1C). These data showed that Lv-shABCC4 efficiently knocked down the expression of ABCC4 in MGC80-3 gastric cancer cells.

Knockdown of ABCC4 inhibited gastric cancer cell growth

To clarify the effects of ABCC4 on gastric cancer cell growth, we conducted MTT cell viability assay and plate colony formation assay. Cell proliferation of untranfected and transfected MGC80-3 cells was measured by MTT assay once daily for 5 days. We found the number of viable cells in control and Lv-shCon groups was similar while viable cells in Lv-shABCC4 group was notably reduced (***p<0.001, Figure 2). Moreover, MGC80-3 cells failed to form colonies after infection with Lv-shABCC4, while the colony numbers in Lv-shCon group were as many as in control group (Figure

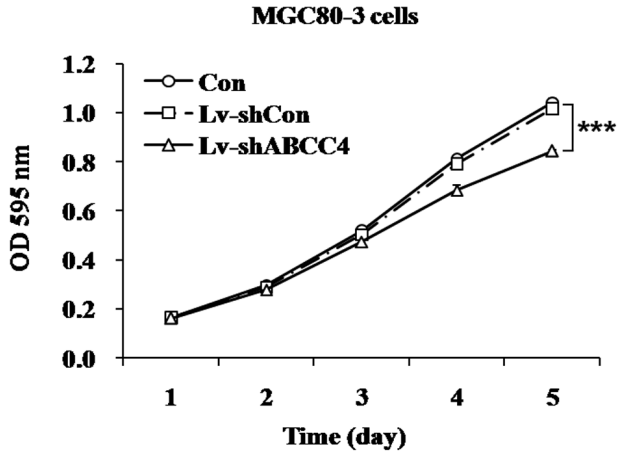

Figure 2. Knockdown of ABCC4 inhibited the proliferation of MGC80-3 cells. Cell proliferation of untranfected or transfected MGC80-3 cells was measured by MTT assay once daily for 5 days. Data represent the mean ± SD of three independent experiments. ***p<0.001, compared with Lv-shCon.

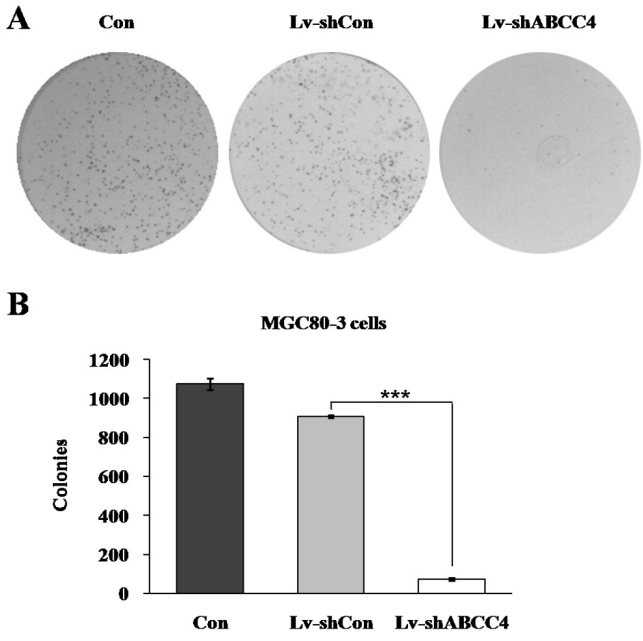

Figure 3. Knockdown of ABCC4 suppressed colony formation of MGC80-3 cells. (A) MGC80-3 cells were seeded at 1000 per well and allowed to form colonies. The colonies stained with Giemsa were observed under light microscopy. (B) Colony numbers were counted and recorded. Data represent the mean ± SD of three independent experiments. ***p<0.001, compared with Lv-shCon.

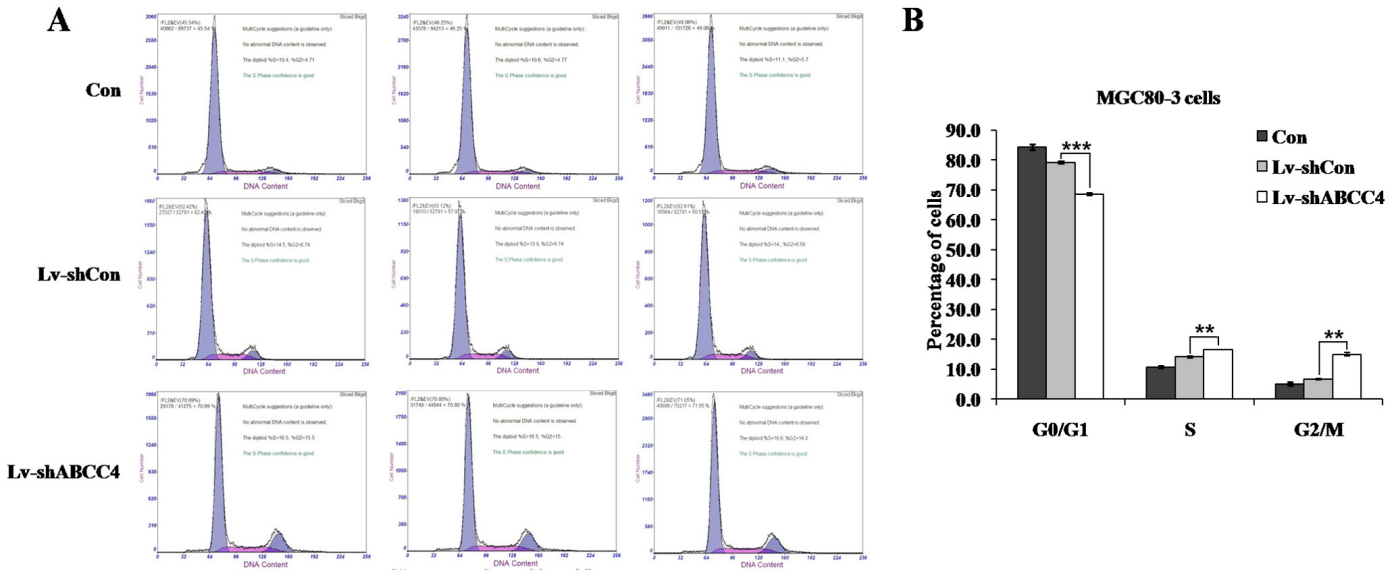

Figure 4. Knockdown of ABCC4 blocked cell cycle progression in MGC80-3 cells. (A) Cell cycle distribution in three distinct groups (control, Lv-shCon and Lv-shABCC4) by flow cytometry analysis. (B) After Lv-shABCC4 infection, the percentage of cells was decreased in G0/G1 phase while increased in S phase and G2/M phase. Data represent the mean ± SD of three independent experiments. ***p*<0.01, ****p*<0.001, compared with Lv-shCon.

3A). The colony formation ability of MGC80-3 cells was remarkably decreased after Lv-shABCC4 infection (***p<0.001, Figure 3B). Therefore, knockdown of ABCC4 was able to inhibit the growth of gastric cancer cells.

Depletion of ABCC4 induced cell cycle arrest in gastric cancer cells

To investigate the mechanism of ABCC4 depletion induced cell proliferation inhibition, flow cytometry analysis of cell cycle was performed. As shown in Figure 4A, MGC80-3 cells in each stage (G0/G1, S or G2/M phase) were differentially distributed in three independent groups (Control, Lv-shCon or Lv-shABCC4 group). After Lv-shABCC4 infection, 68.50% of MGC80-3 cells were in the G0/G1 phase, 16.53% were in the S phase, and 14.93% in the G2/M phase of cell cycle, while 79.17% of Lv-shCon infected cells were in the G0/G1, 14.13% were in the S phase and 6.69% in the G2/M phase of cell cycle. In each phase, cells were similarly distributed in both control and Lv-shCon groups (Figure 4B). Knockdown of ABCC4 induced cell cycle arrest in S phase and G2/M phase (**p<0.01). These results suggested that ABCC4 could modulate the growth of gastric cancer cells via cell cycle regulation.

Discussion

Gastric carcinogenesis is a complex and multifactorial process, in which infection with Helicobacter pylori, environmental factors as well as genetic susceptibility factors play major roles (13). Gastric cancer development results from the accumulation of multiple genetic and epigenetic changes during the lifetime of the patient that will eventually trigger extracellular signals into intracellular signals. As most gastric cancer patients present with late-stage disease and have poor overall survival, the discovery of proteins with aberrant expression are crucially required.

ABC transporters belong to a superfamily that is known to play a crucial role in the development of drug resistance (14). Recently, abnormalities of the members of ABC superfamily transporter genes have also been reported (15, 16). For instance, the relative mRNA levels of ATP-binding cassette, sub-family B 1 (ABCB1), ABCC1, and ABCC3 were highly expressed in cancerous colorectal tissues compared with noncancerous tissues (15). The majority of studied ABCs were down-regulated or unchanged between tumors and control tissues while ABCB6, ABCC, and ABCC2 were up-regulated in tumors versus control tissues (14). Clinical colorectal cancers exhibited substantially increased levels of ABCB5 expression, and further investigation showed the ABCB5-expressing tumor cell population was treatment refractory and exhibited resistance to 5-FU-induced apoptosis (17). High expression of ATP-binding cassette, sub-family G 2 (ABCG2) was found in 30% (9/30) colorectal cancer patients with positive lymph nodes, indicating that ABCG2 may be important in the progression and metastasis of colorectal cancer (16). ABCC4 has been shown to reverse cisplatin resistance in gastric cancer cells (18), however, the role of ABCC4 in the development of gastric cancer remains unclear.

In this study, we found that depletion of ABCC4 by lentivirus-delivered shRNA notably reduced the proliferation and colony formation ability of gastric cancer cells. Cell viability assay is always used to assess cell proliferative rate, which is a hallmark of cell numbers. Similarly, colony formation assay is a convenient assay to assess the capability of cell growth in anchorage-independent condition, and is closely related to the in vivo conditions (19). Attenuation of colony formation indicated that knockdown of ABCC4 could impair the anchorage-independent growth of gastric cancer cells. Altogether, our data suggest that ABCC4 may play a key role in gastric cancer cell growth.

The mechanism underlying ABCC4's tumor promoting-effect is that ABCC4 may regulate key molecules which are crucially involved in cell cycle regulation. As we noted, depletion of ABCC4 in MGC80-3 cells led to an abnormal accumulation of cells in the S phase and

G2/M phase. ABCC4 might contribute to gastric cancer cell growth through manipulation of S phase and G2/M phase cell cycle regulators. A typical example is the fact that knockdown of Aurora Kinase A induces G2/M phase accumulation through regulating of bipolar spindle formation (20). More work need to be done to validate our hypothesis.

In conclusion, it is the first report to define ABCC4 as a functional mediator of gastric cancer cell growth. Our study may provide novel clues for development of therapeutic molecules to treat gastric cancers.

Acknowledgements

This study was supported by grants from the Natural Science Foundation of Shanghai of China (12ZR1420100).

References

1. Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., Global cancer statistics. *CA Cancer J Clin* 2011, **61**: 69-90. doi: 10.3322/caac.20107.

2. Guo, P., Huang, Z.L., Yu, P., Li, K., Trends in cancer mortality in China: an update. *Ann Oncol* 2012, **23**: 2755-2762. doi: mds069 [pii].

3. Arnold, M., Moore, S.P., Hassler, S., Ellison-Loschmann, L., Forman, D., Bray, F., The burden of stomach cancer in indigenous populations: a systematic review and global assessment. *Gut* 2013. doi: 10.1136/gutjnl-2013-305033.

4. Hartgrink, H.H., Jansen, E.P., van Grieken, N.C., van de Velde, C.J., Gastric cancer. *Lancet* 2009, **374**: 477-490. doi: 10.1016/S0140-6736(09)60617-6.

5. Okines, A., Verheij, M., Allum, W., Cunningham, D., Cervantes, A., Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2010, **21 Suppl 5**: v50-54. doi: 10.1093/annonc/mdq164.

6. Fletcher, J.I., Haber, M., Henderson, M.J., Norris, M.D., ABC transporters in cancer: more than just drug efflux pumps. *Nat Rev Cancer* 2010, **10**: 147-156. doi: 10.1038/nrc2789.

7. Keppler, D., Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb Exp Pharmacol*. 2011, 299-323. doi: 10.1007/978-3-642-14541-4_8.

8. van Aubel, R.A., Smeets, P.H., Peters, J.G., Bindels, R.J., Russel, F.G., The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol* 2002, **13**: 595-603.

9. Yu, Z.Q., Zhang, C., Wang, H., Lao, X.Y., Chai, R., Gao, X.H., Cao, G.W., and Fu, C.G., Downregulation of ATP-binding cassette subfamily C member 4 increases sensitivity to neoadjuvant radiotherapy for locally advanced rectal carcinoma. *Dis Colon Rectum* 2013, **56**: 600-608. doi: 10.1097/DCR.0b013e31827c2b80.

10. Zhang, Z., Wang, J., Shen, B., Peng, C., Zheng, M., The ABCC4 gene is a promising target for pancreatic cancer therapy. *Gene* 2012, **491**: 194-199. doi: 10.1016/j.gene.2011.09.029.

11. Copsel, S., Garcia, C., Diez, F., Vermeulem, M., Baldi, A., Bianciotti, L.G., Russel, F.G., Shayo, C., and Davio, C., Multidrug resistance protein 4 (MRP4/ABCC4) regulates cAMP cellular levels and controls human leukemia cell proliferation and differentiation. *J Biol Chem* 2011, **286**: 6979-6988. doi: 10.1074/jbc.M110.166868.

12. Advani, A.S., Shadman, M., Ali-Osman, F., Barker, A., Rybicki, L., Kalaycio, M., Sekeres, M.A., de Castro, C.M., Diehl, L.F., Moore, J.O., Beaven, A., Copelan, E., Sobecks, R., Talea, P., and Rizzieri, D.A., A Phase II trial of gemcitabine and mitoxantrone for patients with acute myeloid leukemia in first relapse. *Clin Lymphoma Myeloma Leuk* 2010, **10**: 473-476. doi: 10.3816/CLML.2010.n.082.

13. Guggenheim, D.E., Shah, M.A., Gastric cancer epidemiology and risk factors. *J Surg Oncol* 2013, **107**: 230-236. doi: 10.1002/jso.23262.

14. Hlavata, I., Mohelnikova-Duchonova, B., Vaclavikova, R., Liska, V., Pitule, P., Novak, P., Bruha, J., Vycital, O., Holubec, L., Treska, V., Vodicka, P., and Soucek, P., The role of ABC transporters in progression and clinical outcome of colorectal cancer. *Mutagenesis* 2012, **27**: 187-196. doi: 10.1093/mutage/ger075.

15. Hinoshita, E., Uchiumi, T., Taguchi, K., Kinukawa, N., Tsuneyoshi, M., Maehara, Y., Sugimachi, K., and Kuwano, M., Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clin Cancer Res* 2000, **6**: 2401-2407.

16. Liu, H.G., Pan, Y.F., You, J., Wang, O.C., Huang, K.T., Zhang, X.H., Expression of ABCG2 and its significance in colorectal cancer. *Asian Pacific journal of cancer prevention : APJCP* 2010, **11**: 845-848.

17. Wilson, B.J., Schatton, T., Zhan, Q., Gasser, M., Ma, J., Saab, K.R., Schanche, R., Waaga-Gasser, A.M., Gold, J.S., Huang, Q., Murphy, G.F., Frank, M.H., and Frank, N.Y., ABCB5 identifies a therapy-refractory tumor cell population in colorectal cancer patients. *Cancer Res* 2011, **71**: 5307-5316. doi: 10.1158/0008-5472.CAN-11-0221.

18. Zhang, Y.H., Wu, Q., Xiao, X.Y., Li, D.W., Wang, X.P., Silencing MRP4 by small interfering RNA reverses acquired DDP resistance of gastric cancer cell. *Cancer letters* 2010, **291**: 76-82. doi: 10.1016/j.canlet.2009.10.003.

19. Zhou, C., Ji, J., Cai, Q., Shi, M., Chen, X., Yu, Y., Liu, B., Zhu, Z., and Zhang, J., MTA2 promotes gastric cancer cells invasion and is transcriptionally regulated by Sp1. *Mol Cancer* 2013, **12**: 102. doi: 10.1186/1476-4598-12-102.

20. Yang, G., Chang, B., Yang, F., Guo, X., Cai, K.Q., Xiao, X.S., Wang, H., Sen, S., Hung, M.C., Mills, G.B., Chang, S., Multani, A.S., Mercado-Uribe, I., and Liu, J., Aurora kinase A promotes ovarian tumorigenesis through dysregulation of the cell cycle and suppression of BRCA2. *Clin Cancer Res* 2010, **16**: 3171-3181. doi: 10.1158/1078-0432.CCR-09-3171.