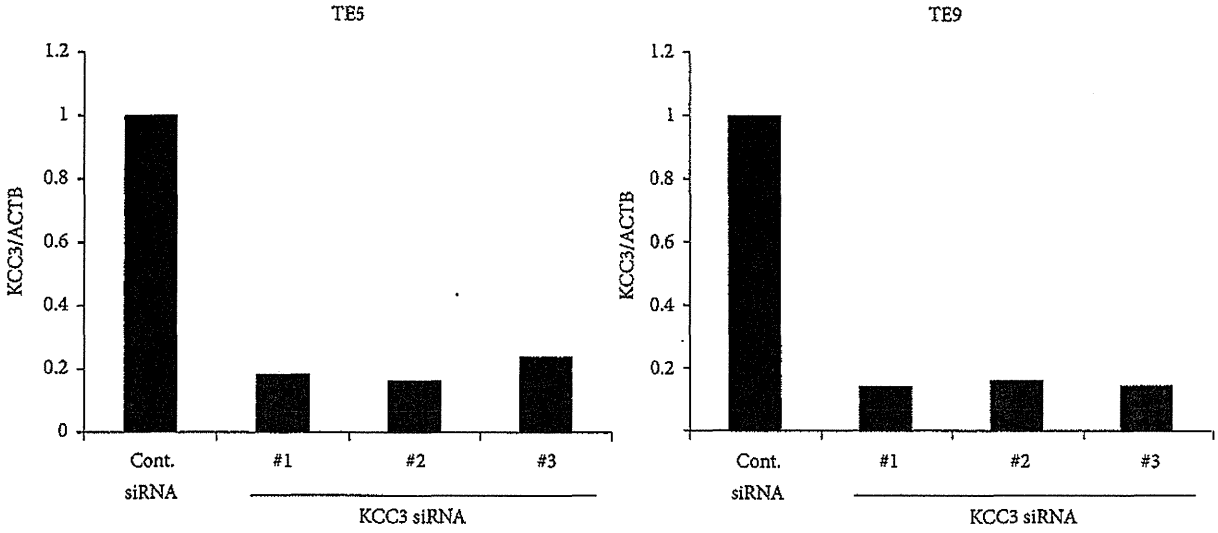
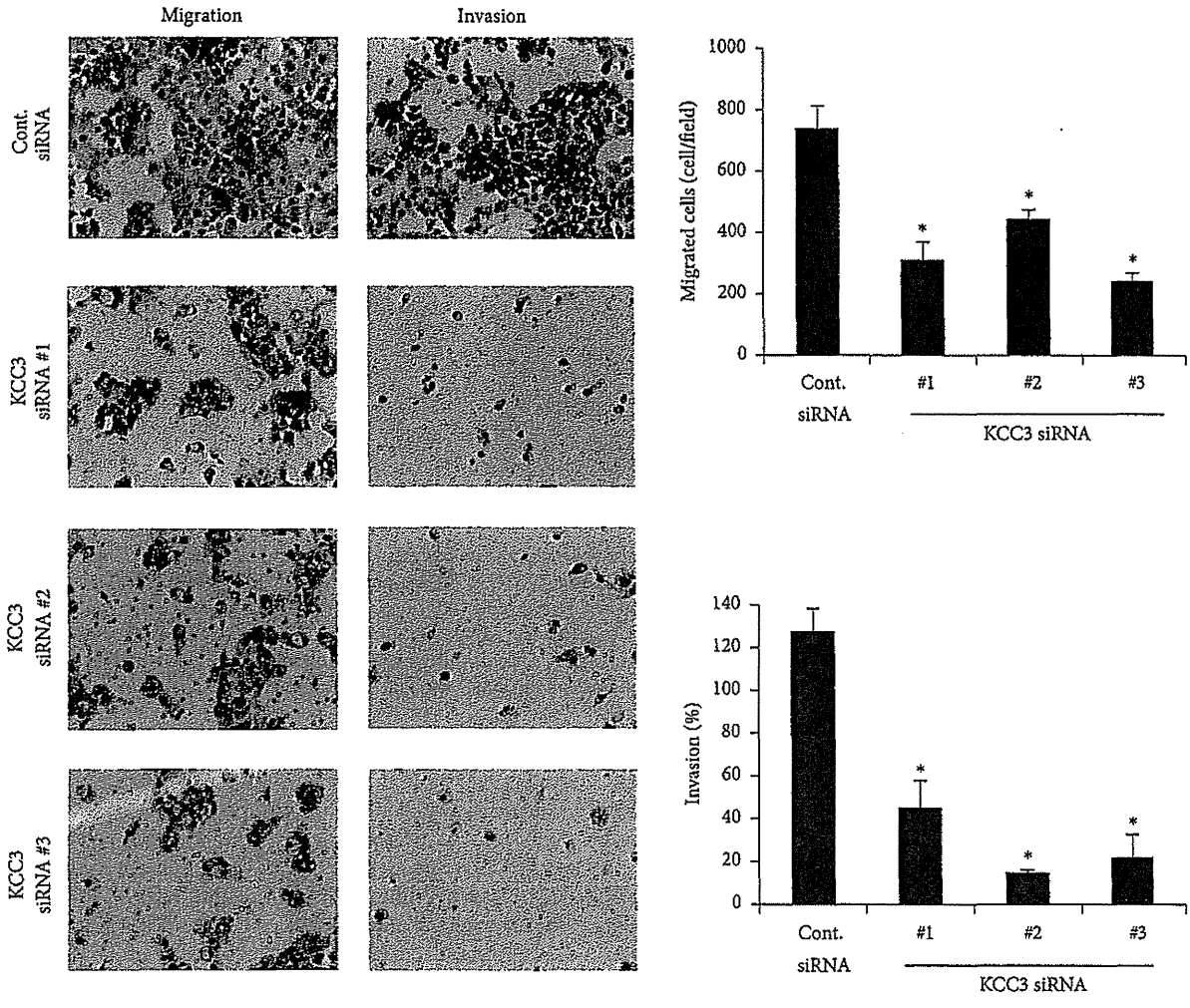


(a)



(b)

FIGURE 4: Continued.

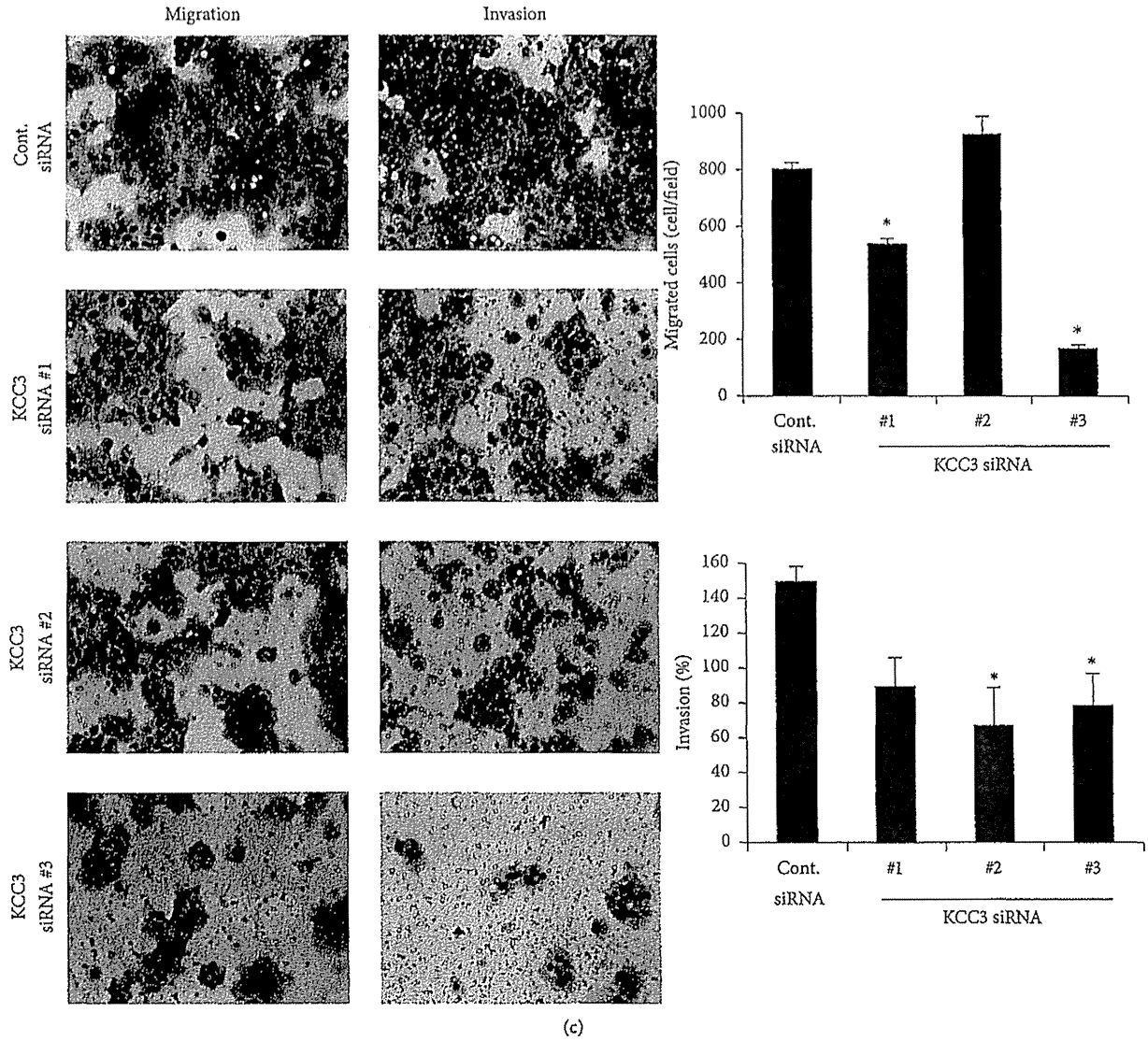


FIGURE 4: KCC3 controlled the cell migration and invasion of esophageal squamous cell carcinoma (ESCC) cells. (a) KCC3 siRNA effectively reduced KCC3 mRNA levels in both TE5 and TE9 cells. Three independent KCC3 siRNAs were investigated to exclude off target effects. (b) The downregulation of KCC3 significantly inhibited cell migration and invasion in TE5 cells. Cell migration and invasion were determined by the Boyden chamber assay. Mean ± SEM; $n = 3$. $^*P < 0.05$: Dunnett's test (ANOVA: migration; $P < 0.0001$, invasion; $P < 0.0001$). (c) The downregulation of KCC3 inhibited cell migration and invasion in TE9 cells. Cell migration and invasion were determined by the Boyden chamber assay. Mean ± SEM; $n = 3$. $^*P < 0.05$: Dunnett's test (ANOVA: migration; $P < 0.0001$, invasion; $P = 0.0210$).

roles in the tumorigenesis of colorectal, gastric, cervical, breast, lung, and prostate cancer cells [17, 18, 26]. We have also focused on and investigated transepithelial Cl^- transport in various types of cancer cells [11, 27–29].

In the present study, we investigated the KCC3 expression in ESCC and determined its relationships with clinicopathological features and prognosis. To the best of our knowledge, this is the first report examining KCC3 expression in human ESCC tissue. Our results showed that KCC3 expression in MT related to several clinicopathological features, such as the pT and pN categories. However, the expression of KCC3 in MT itself did not have a prognostic impact. Although these results may not be persuasive because of the limitation of a small sample size, they showed that KCC3 was expressed in MT of ESCC from an early stage. Regarding the expression of KCC in cancer tissue, previous studies demonstrated that KCC3 was abundant in cervical carcinoma and CN invaded deeply into stromal tissues whereas KCC4 was abundant in metastatic cervical and ovarian cancer tissues [8, 10]. Furthermore, both the progression-free and overall survival rates of patients with the high grade expression of KCC4 were significantly poorer than those of patients with the low grade expression of KCC4 in cervical cancer [10], which suggested a relationship between the expression pattern of KCC and

Table 2: Relationships between the clinicopathological features of esophageal cancer and expression of KCC3 in the invasive front of the tumor.

Variable	Invasive front		P value
	Negative ($n = 22$)	Positive ($n = 48$)	
Age			
<60 years	6	16	0.783
≥60 years	16	32	
Gender			
Male	21	38	0.154
Female	1	10	
Location of the primary tumor			
Ut-Mt	12	35	0.172
Lt-Ae	10	13	
Histological type			
Well/moderately differentiated SCC	15	34	1.000
Poorly differentiated SCC	7	14	
Tumor size			
<50 mm	14	35	0.575
≥50 mm	8	13	
Lymphatic invasion			
Negative	10	23	1.000
Positive	12	25	
Venous invasion			
Negative	13	27	1.000
Positive	9	21	
pT			
pT1	8	25	0.3035
pT2-3	14	23	
pN			
pN0	8	25	0.3035
pN1-3	14	23	
MT			
Low	20	15	<0.0001*
High	2	33	
CN			
Low	19	17	<0.0001*
High	3	31	
CN/MT			
CN ≤ MT	14	17	0.0385*
CN > MT	8	31	

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; SCC: squamous cell carcinoma; pT: pathological T stage; pN: pathological N stage; MT: main tumor; CN: cancer nest.
*$P < 0.05$: Fisher's exact test.

Table 3: Five-year survival rate of patients with esophageal cancer according to various clinicopathological parameters.

Variables	5-year survival rate (%)	P value
Age		
<60 years	53.13	0.1179
≥60 years	72.2	
Gender		
Male	65.35	0.8597
Female	68.57	
Location of the primary tumor		
Ut-Mt	73.63	0.0564
Lt-Ae	50.82	
Histological type		
Well/moderately differentiated SCC	71.94	0.2301
Poorly differentiated SCC	53.43	
Tumor size		
<50 mm	68.68	0.2809
≥50 mm	59.52	
Lymphatic invasion		
Negative	78.4	0.0168*
Positive	54.78	
Venous invasion		
Negative	78.18	0.0169*
Positive	48.7	
pT		
pT1	82.22	0.0024*
pT2-3	50.68	
pN		
pN0	82.72	0.0029*
pN1-3	49.68	
MT		
Low	68.21	0.7838
High	60.56	
CN		
Low	70.48	0.4151
High	59.25	
CN/MT		
CN ≤ MT	76.74	0.1329
CN > MT	55.48	
Invasive front		
Negative	81.82	0.0887
Positive	57.07	

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; SCC: squamous cell carcinoma; pT: pathological T stage; pN: pathological N stage; MT: main tumor; CN: cancer nest.
*$P < 0.05$: log-rank test.

clinical outcome. Therefore, we focused on the distribution of KCC3 in tumors and analyzed its expression in CN or the invasive front of the tumor. Although the expression of KCC3 in CN itself had no prognostic impact, the 5-year survival rate of patients with a CN > MT score was slightly lower than that of patients with a CN ≤ MT score. Furthermore, the 5-year survival rate of patients in whom KCC3 was expressed in the invasive front was lower than that of patients without it, and multivariate analysis revealed that

Table 4: Prognostic factors of esophageal cancer according to multivariate analysis.

Variables	Risk ratio	95% CI	*P* value
Location of the primary tumor			
Ut-Mt	Ref.	0.861452–2.20629	0.1813
Lt-Ae	1.37267		
Lymphatic invasion			
Negative	Ref.	1.012886–2.725895	0.0437*
Positive	1.605417		
Venous invasion			
Negative	Ref.	0.954510–2.395284	0.08
Positive	1.483897		
pT			
pT1	Ref.	1.122531–3.250204	0.0146*
pT2-3	1.834223		
pN			
pN0	Ref.	1.152392–3.450263	0.0110*
pN1-3	1.911249		
Invasive front			
Negative	Ref.	1.357757–4.524146	0.0014*
Positive	2.332559		

Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; pT: pathological T stage; pN: pathological N stage; Ref.: referent.
*$P < 0.05$: Cox's proportional hazards model; 95% CI: 95% confidence interval.

the expression of KCC3 in the invasive front was the strongest prognostic factor of all clinicopathological features. These results suggest the role of KCC3 in cancer invasion as well as the importance of its distribution in tumors as a prognostic predictor. We have previously identified several prognostic biomarkers in human ESCC, such as Ki-67, antiphosphohistone H3, p21, and E2F5 [30–33]. The expressions of these cell-cycle related proteins were mainly analyzed in MT. On the other hand, we focused on the distribution of KCC3 in the present study and showed its prognostic impact via cellular invasion.

Recent studies have indicated the importance of KCC in the cell migration and invasion of glioma, cervical, ovarian, and breast cancer cells [8–10, 12, 13]. Regarding the mechanism by which KCC regulates tumor invasion, KCC3 was previously shown to downregulate the formation of the E-cadherin/β-catenin complex in order to promote EMT, which is important for cervical cancer cell invasiveness [8]. In addition, a previous study reported that the motor protein-dependent membrane trafficking of KCC4 was important for cancer cell invasion [10]. Our *in vitro* study also demonstrated the important roles of KCC3 in cell migration and invasion in ESCC cells. One possible mechanism by which KCC regulates the malignant behavior of cancer cells may be through the regulation of $[Cl^-]_i$ [11, 13]. Recent studies have shown that $[Cl^-]_i$ is a critical signal mediator for the regulation of various cellular functions [34–36]. For instance, we showed that $[Cl^-]_i$ could act as an important signal to control the gene expression of the epithelial Na^+ channel via a tyrosine kinase in renal epithelial A6 cells [36]. We also previously reported that $[Cl^-]_i$ controlled cell-cycle progression in gastric and prostate cancer cells [27–29, 37, 38]. Shen et al. showed that an alteration in the $[Cl^-]_i$ concentration affected the activity of the retinoblastoma protein and cdc2 kinase, two key cell-cycle regulators that control progression from the G_1 into the S phase and from the G_2 into the M phase, respectively [13]. We considered KCC to be one of the important transporters that regulates $[Cl^-]_i$ in the steady state and previously showed that the blockage of KCC decreased $[Cl^-]_i$ in breast cancer cells [11]. Although this mechanism should be verified in more detail in further studies, these findings suggest that the changes induced in $[Cl^-]_i$ by KCC3 may be a critically important messenger that regulates cellular invasiveness in ESCC cells.

In summary, we found that KCC3 played a role in the cell migration and invasion of ESCC cells. An immunohistochemical analysis revealed that the expression of KCC3 in the invasive front of tumors was the strongest prognostic factor in patients with ESCC. A deeper understanding of the role of KCC3 may lead to its use as a crucial biomarker of tumor progression and/or a new therapeutic target for ESCC.

Conflict of Interests

None of the authors have any conflict of interests or financial ties to disclose.

Authors' Contribution

Atsushi Shiozaki and Kenichi Takemoto contributed equally to this work.

Acknowledgments

This work was supported by Grants-in-Aid for Young Scientists (B) (22791295, 24791440) and a Grant-in-Aid for Scientific Research (C) (22591464, 24591957, and 26461988) from the Japan Society for the Promotion of Science.

References

[1] P. K. Lauf and N. C. Adragna, "K-Cl cotransport: properties and molecular mechanism," *Cellular Physiology and Biochemistry*, vol. 10, no. 5-6, pp. 341–354, 2000.

[2] P. B. Dunham, G. W. Stewart, and J. C. Ellory, "Chloride-activated passive potassium transport in human erythrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 3, pp. 1711–1715, 1980.

[3] Y. F. Chen, C. Y. Chou, J. C. Ellory, and M. R. Shen, "The emerging role of KCl cotransport in tumor biology," *The American Journal of Translational Research*, vol. 2, no. 4, pp. 345–355, 2010.

[4] D. B. Mount, A. Mercado, L. Song et al., "Cloning and characterization of KCC3 and KCC4, new members of the cation-chloride cotransporter gene family," *The Journal of Biological Chemistry*, vol. 274, no. 23, pp. 16355–16362, 1999.

[5] C. M. Gillen, S. Brill, J. A. Payne, and B. Forbush III, "Molecular cloning and functional expression of the K-Cl cotransporter from rabbit, rat, and human: A new member of the cation-chloride cotransporter family," *Journal of Biological Chemistry*, vol. 271, no. 27, pp. 16237–16244, 1996.

[6] J. A. Payne, T. J. Stevenson, and L. F. Donaldson, "Molecular characterization of a putative K-Cl cotransporter in rat brain: a neuronal-specific isoform," *Journal of Biological Chemistry*, vol. 271, no. 27, pp. 16245–16252, 1996.

[7] J. E. Race, F. N. Makhlouf, P. J. Logue, F. H. Wilson, P. B. Dunham, and E. J. Holtzman, "Molecular cloning and functional characterization of KCC3, a new K-Cl cotransporter," *The American Journal of Physiology*, vol. 277, no. 6, part 1, pp. C1210–C1219, 1999.

[8] Y. M. Hsu, Y. F. Chen, C. Y. Chou et al., "KCl cotransporter-3 down-regulates E-cadherin/β-catenin complex to promote epithelial-mesenchymal transition," *Cancer Research*, vol. 67, no. 22, pp. 11064–11073, 2007.

[9] Y. M. Hsu, C. Y. Chou, H. H. Chen et al., "IGF-1 upregulates electroneutral K-Cl cotransporter KCC3 and KCC4 which are differentially required for breast cancer cell proliferation and invasiveness," *Journal of Cellular Physiology*, vol. 210, no. 3, pp. 626–636, 2007.

[10] Y. F. Chen, C. Y. Chou, R. J. Wilkins, J. C. Ellory, D. B. Mount, and M. Shen, "Motor protein-dependent membrane trafficking of KCl cotransporter-4 is important for cancer cell invasion," *Cancer Research*, vol. 69, no. 22, pp. 8585–8593, 2009.

[11] M. Kitagawa, N. Niisato, A. Shiozaki et al., "A regulatory role of K^+-Cl^- cotransporter in the cell cycle progression of breast cancer MDA-MB-231 cells," *Archives of Biochemistry and Biophysics*, vol. 539, no. 1, pp. 92–98, 2013.

[12] K. B. Gagnon, "High-grade glioma motility reduced by genetic knockdown of KCC3," *Cellular Physiology and Biochemistry*, vol. 30, no. 2, pp. 466–476, 2012.

[13] M. R. Shen, C. Y. Chou, K. F. Hsu et al., "The KCl cotransporter isoform KCC3 can play an important role in cell growth regulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 25, pp. 14714–14719, 2001.

[14] L. Sobin, M. Gospodarowicz, and C. Wittekind, Eds., *TNM Classification of Malignant Tumors*, John Wiley & Sons, Hoboken, NJ, USA, 7th edition, 2009.

[15] W. Remmele and H. E. Stegner, "Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue," *Pathologe*, vol. 8, no. 3, pp. 138–140, 1987.

[16] T. Nishihira, Y. Hashimoto, M. Katayama, S. Mori, and T. Kuroki, "Molecular and cellular features of esophageal cancer cells," *Journal of Cancer Research and Clinical Oncology*, vol. 119, no. 8, pp. 441–449, 1993.

[17] K. Kunzelmann, "Ion channels and cancer," *Journal of Membrane Biology*, vol. 205, no. 3, pp. 159–173, 2005.

[18] R. Schönherr, "Clinical relevance of ion channels for diagnosis and therapy of cancer," *The Journal of Membrane Biology*, vol. 205, no. 3, pp. 175–184, 2005.

[19] C. J. Kim, Y. G. Cho, S. W. Jeong et al., "Altered expression of KCNK9 in colorectal cancers," *APMIS*, vol. 112, no. 9, pp. 588–594, 2004.

[20] X. D. Shao, K. C. Wu, Z. M. Hao, L. Hong, J. Zhang, and D. Fan, "The potent inhibitory effects of cisapride, a specific blocker for human ether-a-go-go-related gene (HERG) channel, on gastric cancer cells," *Cancer Biology and Therapy*, vol. 4, no. 3, pp. 295–301, 2005.

[21] X. Wang, Y. Nagaba, H. S. Cross, F. Wrba, L. Zhang, and S. E. Guggino, "The mRNA of L-type calcium channel elevated in colon cancer: protein distribution in normal and cancerous colon," *The American Journal of Pathology*, vol. 157, no. 5, pp. 1549–1562, 2000.

[22] R. Cai, X. Ding, K. Zhou et al., "Blockade of TRPC6 channels induced G2/M phase arrest and suppressed growth in human gastric cancer cells," *International Journal of Cancer*, vol. 125, no. 10, pp. 2281–2287, 2009.

[23] J. Wu, Y.-C. Zhang, W.-H. Suo et al., "Induction of anion exchanger-1 translation and its opposite roles in the carcinogenesis of gastric cancer cells and differentiation of K562 cells," *Oncogene*, vol. 29, no. 13, pp. 1987–1996, 2010.

[24] H. Nagata, X. Che, K. Miyazawa et al., "Rapid decrease of intracellular pH associated with inhibition of Na +/H+ exchanger precedes apoptotic events in the MNK45 and MNK74 gastric cancer cell lines treated with 2-aminophenoxazine-3-one," *Oncology Reports*, vol. 25, no. 2, pp. 341–346, 2011.

[25] J. Chen, C. Röcken, J. Hoffmann et al., "Expression of carbonic anhydrase 9 at the invasion front of gastric cancers," *Gut*, vol. 54, no. 7, pp. 920–927, 2005.

[26] C. D. Chen, C. S. Wang, Y. H. Huang et al., "Overexpression of CLIC1 in human gastric carcinoma and its clinicopathological significance," *Proteomics*, vol. 7, no. 1, pp. 155–167, 2007.

[27] A. Shiozaki, E. Otsuji, and Y. Marunaka, "Intracellular chloride regulates the G1/S cell cycle progression in gastric cancer cells," *World Journal of Gastrointestinal Oncology*, vol. 3, no. 8, pp. 119–122, 2011.

[28] H. Miyazaki, A. Shiozaki, N. Niisato et al., "Chloride ions control the G1/S cell-cycle checkpoint by regulating the expression of p21 through a p53-independent pathway in human gastric cancer cells," *Biochemical and Biophysical Research Communications*, vol. 366, no. 2, pp. 506–512, 2008.

[29] R. Ohsawa, H. Miyazaki, N. Niisato et al., "Intracellular chloride regulates cell proliferation through the activation of stress-activated protein kinases in MKN28 human gastric cancer cells," *Journal of Cellular Physiology*, vol. 223, no. 3, pp. 764–770, 2010.

[30] H. Sasagawa, A. Shiozaki, D. Iitaka et al., "Ki-67 labeling index as an independent prognostic factor in human esophageal squamous cell carcinoma," *Esophagus*, vol. 9, no. 4, pp. 195–202, 2012.

[31] S. Nakashima, A. Shiozaki, D. Ichikawa et al., "Anti-phosphohistone H3 as an independent prognostic factor in human esophageal squamous cell carcinoma," *Anticancer Research*, vol. 33, no. 2, pp. 461–467, 2013.

[32] A. Shiozaki, S. Nakashima, D. Ichikawa et al., "Prognostic significance of p21 expression in patients with esophageal squamous cell carcinoma," *Anticancer Research*, vol. 33, no. 10, pp. 4329–4335, 2013.

[33] T. Ishimoto, A. Shiozaki, D. Ichikawa et al., "E2F5 as an independent prognostic factor in esophageal squamous cell carcinoma," *Anticancer Research*, vol. 33, no. 12, pp. 5415–5420, 2013.

[34] B. Jiang, N. Hattori, B. Liu et al., "Expression and roles of Cl-channel ClC-5 in cell cycles of myeloid cells," *Biochemical and Biophysical Research Communications*, vol. 317, no. 1, pp. 192–197, 2004.

[35] R. Menegazzi, S. Busetto, P. Dri, R. Cramer, and P. Patriarca, "Chloride ion efflux regulates adherence, spreading, and respiratory burst of neutrophils stimulated by tumor necrosis factor-α (TNF) on biologic surfaces," *Journal of Cell Biology*, vol. 135, no. 2, pp. 511–522, 1996.

[36] N. Niisato, D. C. Eaton, and Y. Marunaka, "Involvement of cytosolic Cl^- in osmoregulation of α-ENaC gene expression," *The American Journal of Physiology: Renal Physiology*, vol. 287, no. 5, pp. F932–F939, 2004.

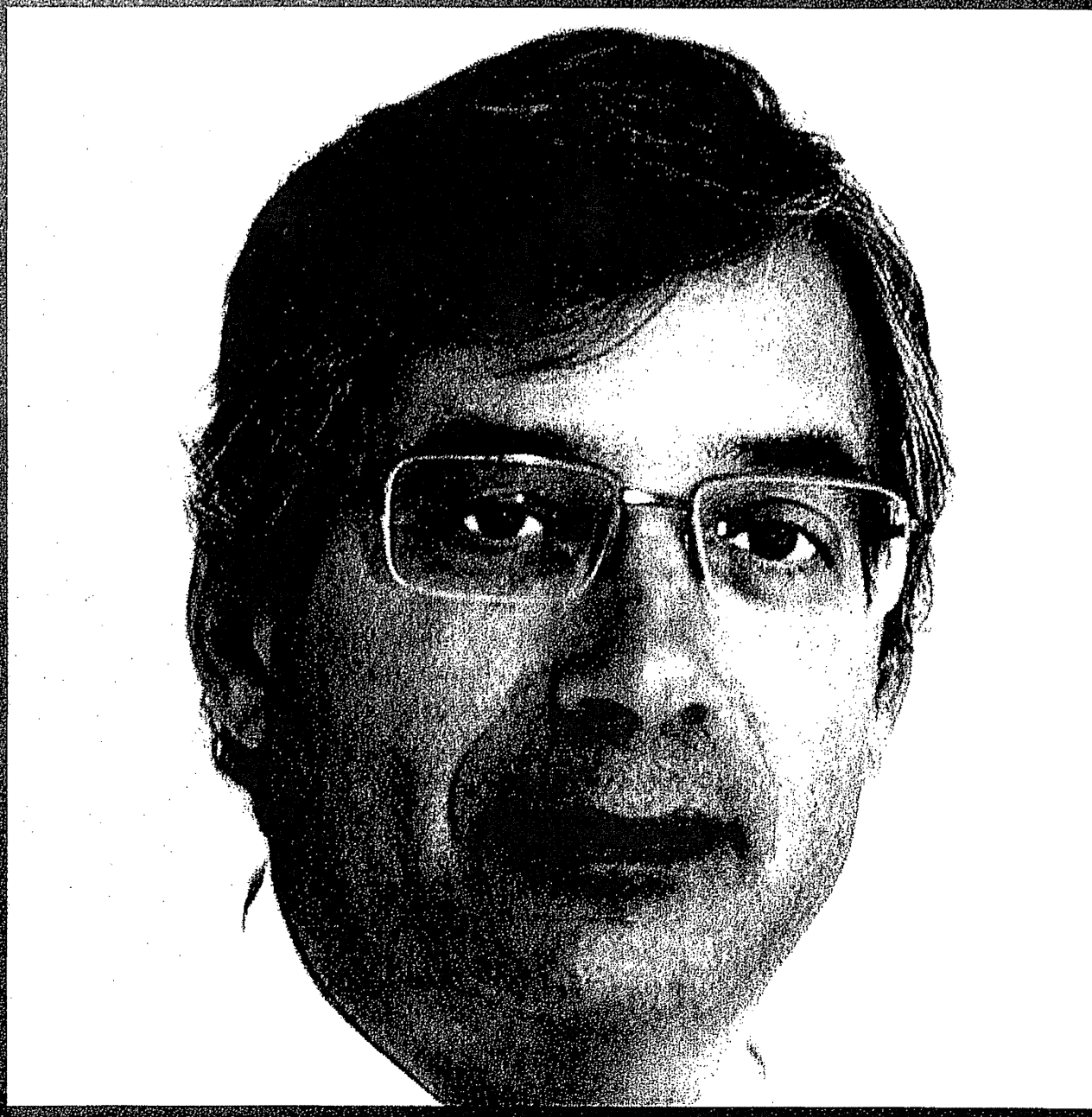
[37] A. Shiozaki, H. Miyazaki, N. Niisato et al., "Furosemide, a blocker of $Na^+/K^+/2Cl^-$ cotransporter, diminishes proliferation of poorly differentiated human gastric cancer cells by affecting G0/G1 state," *Journal of Physiological Sciences*, vol. 56, no. 6, pp. 401–406, 2006.

[38] K. Hiraoka, H. Miyazaki, N. Niisato et al., "Chloride ion modulates cell proliferation of human androgen-independent prostatic cancer cell," *Cellular Physiology and Biochemistry*, vol. 25, no. 4-5, pp. 379–388, 2010.

ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of Gastroenterology

World J Gastroenterol 2016 June 14; 22(22): 5201-5203



Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 20 Number 22 June 14, 2014

TOPIC HIGHLIGHT

Contents

World Journal of Gastroenterology
Volume 20 Number 22 June 14, 2014

World Journal of Gastroenterology
Volume 20 Number 22 June 14, 2014

Contents

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2012 Impact Factor: 2.547 (34/74); Total Cites: 19145 (6/74); Current Articles: 944 (1/74); and Eigenfactor® Score: 0.06035 (6/74).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
June 14, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i22.6844

World J Gastroenterol 2014 June 14; 20(22): 6844-6859
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Role of the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 in cell cycle progression in human esophageal squamous cell carcinoma

Atsushi Shiozaki, Yoshito Nako, Daisuke Ichikawa, Hirotaka Konishi, Shuhei Komatsu, Takeshi Kubota, Hitoshi Fujiwara, Kazuma Okamoto, Mitsuo Kishimoto, Yoshinori Marunaka, Eigo Otsuji

Atsushi Shiozaki, Yoshito Nako, Daisuke Ichikawa, Hirotaka Konishi, Shuhei Komatsu, Takeshi Kubota, Hitoshi Fujiwara, Kazuma Okamoto, Eigo Otsuji, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
Mitsuo Kishimoto, Department of Pathology, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
Yoshinori Marunaka, Departments of Molecular Cell Physiology and Bio-Ionomics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
Yoshinori Marunaka, Japan Institute for Food Education and Health, St. Agnes' University, Kyoto 602-8013, Japan
Author contributions: Shiozaki A and Nako Y contributed equally to this work; Shiozaki A and Nako Y designed the study, wrote the manuscript and performed the majority of the experiments; Ichikawa D, Konishi H, Komatsu S, Kubota T, Fujiwara H, Okamoto K and Kishimoto M collected and analyzed the clinicopathological data obtained from ESCC patients; Marunaka Y and Otsuji E were involved in editing the manuscript.
Supported by Grants-in-Aid for Young Scientists (B), NO. 22791295, NO. 23791557, and NO. 24791440; and a Grant-in-Aid for Scientific Research (C), NO.22591464 and NO. 24591957, from the Japan Society for the Promotion of Science
Correspondence to: Atsushi Shiozaki, Assistant Professor, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan. shiozaki@koto.kpu-m.ac.jp
Telephone: +81-75-2515527 Fax: +81-75-2515522
Received: October 28, 2013 Revised: January 17, 2014
Accepted: February 17, 2014
Published online: June 14, 2014

Abstract

AIM: To investigate the role of $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) in the regulation of genes involved in cell cycle progression and the clinicopathological significance of its expression in esophageal squamous cell carcinoma (ESCC).

METHODS: An immunohistochemical analysis was performed on 68 primary tumor samples obtained from ESCC patients that underwent esophagectomy. NKCC1 expression in human ESCC cell lines was analyzed by Western blotting. Knockdown experiments were conducted using NKCC1 small interfering RNA, and the effects on cell cycle progression were analyzed. The gene expression profiles of cells were analyzed by microarray analysis.

RESULTS: Immunohistochemical staining showed that NKCC1 was primarily found in the cytoplasm of carcinoma cells and that its expression was related to the histological degree of differentiation of SCC. NKCC1 was highly expressed in KYSE170 cells. Depletion of NKCC1 in these cells inhibited cell proliferation *via* G_2/M phase arrest. Microarray analysis identified 2527 genes with altered expression levels in NKCC1depleted KYSE170. Pathway analysis showed that the top-ranked canonical pathway was the G_2/M DNA damage checkpoint regulation pathway, which involves MAD2L1, DTL, BLM, CDC20, BRCA1, and E2F5.

CONCLUSION: These results suggest that the expression of NKCC1 in ESCC may affect the G_2/M checkpoint and may be related to the degree of histological differentiation of SCCs. We have provided a deeper understanding of the role of NKCC1 as a mediator and/or a biomarker in ESCC.

Key words: $Na^+/K^+/2Cl^-$ cotransporter 1; Esophageal cancer; Cell cycle

Core tip: The objectives of the present study were to investigate the role of $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) in the regulation of genes involved in cell cycle progression and the clinicopathological significance

of its expression in esophageal squamous cell carcinoma (ESCC). An immunohistochemical analysis revealed that the expression of NKCC1 in ESCC samples was related to the histological type. Microarray results suggested that NKCC1 exhibits marked effects on the expression of genes related to G2/M cell cycle progression. A deeper understanding of the role of NKCC1 may lead to its use as an important biomarker and/or a novel therapeutic target for ESCC treatment.

Shiozaki A, Nako Y, Ichikawa D, Konishi H, Komatsu S, Kubota T, Fujiwara H, Okamoto K, Kishimoto M, Marunaka Y, Otsuji E. Role of the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 in cell cycle progression in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2014; 20(22): 6844-6859 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i22/6844.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i22.6844

INTRODUCTION

Several studies have recently shown that ion channels and transporters play important roles in fundamental cellular functions. Their physiological roles in cell proliferation have been studied in more detail because ion transport across the cell membrane is involved in the regulation of cell volume, which is indispensable for cell cycle progression. Several reports have demonstrated the important roles of Cl^- channels/transporters, such as Ca^{2+}- activated $2Cl^-$ channels and Cl^-/HCO^{3-} exchangers, in gastrointestinal cancer cells[1,2]. These studies indicated that transepithelial Cl^- transport plays an important role in the proliferation of gastrointestinal cancer cells.

The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is a member of the cation-chloride cotransporter family. NKCC transports one sodium ion, one potassium ion, and two chloride ions across the plasma membrane and is sensitive to loop diuretics, such as furosemide and bumetanide. There are two isoforms of NKCC, and NKCC1 is ubiquitously expressed in various types of cells including epithelial cells[3,4]. We previously examined transepithelial Cl^- transport in various types of cancer cells[5-7] and showed that NKCC1 plays an important role in the proliferation of gastric and prostate cancer cells[8,9]. However, the role of NKCC1 in the proliferation of esophageal squamous cell carcinoma (ESCC) cells and its detailed regulatory mechanisms have not been fully investigated. Furthermore, the clinicopathological meaning of NKCC1 expression in ESCCs remains uncertain.

The objectives of the present study were to investigate the role of NKCC1 in the regulation of genes involved in cell cycle progression and the clinicopathological significance of its expression in ESCC. We analyzed the expression of NKCC1 in human ESCC samples and determined its relationship with the degree of histological differentiation of SCC samples. Furthermore, microarray analyses showed that depletion of NKCC1 with small interfering RNA (siRNA) changed the expression levels of many genes involved in G2/M cell cycle progression. Our results indicate that NKCC1 plays an important role in the tumor progression of ESCCs.

MATERIALS AND METHODS

Cell lines, antibodies, and other reagents

The human ESCC cell lines TE2, TE5, TE9, and TE13 were obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan)[10]. The human ESCC cell lines KYSE70 and KYSE170 were obtained from Kyoto University (Kyoto, Japan)[11]. These cells were grown in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. Cells were cultured in flasks or dishes in a humidified incubator at 37 ℃ under 5% CO_2 in air.

The anti-NKCC1 antibody used for immunohistochemical analysis and the protein assay were obtained from Sigma-Aldrich (St. Louis, MO). The anti-Ki-67 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology. Furosemide was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Patients and primary tissue samples

ESCC tumor samples were obtained from 68 patients with a histologically confirmed primary ESCC who underwent esophagectomy at Kyoto Prefectural University of Medicine between 1998 and 2007 and were embedded in paraffin after 12 h of formalin fixation. Patient eligibility criteria were as follows: no synchronous or metachronous cancers (in addition to ESCC) and no preoperative chemotherapy or radiation therapy. We excluded patients with non-curative resected tumors or non-consecutive data. All patients provided written informed consent. Relevant clinicopathological and survival data were obtained from the hospital database. Staging was principally based on the International Union Against Cancer/tumor node metastasis Classification of Malignant Tumors (7th edition)[12].

Immunohistochemistry

Paraffin sections (4 μm thick) of tumor tissues were subjected to immunohistochemical staining for the NKCC1 protein using the avidin-biotin-peroxidase method. Briefly, paraffin sections were dewaxed with xylene and dehydrated with a graded series of alcohols. Antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (Glostrup, Denmark) for 40 min at 98 ℃. Endogenous peroxidases were quenched by incubating the sections for 30 min in 0.3% H_2O_2. Sections were then treated with protein blocker and incu-

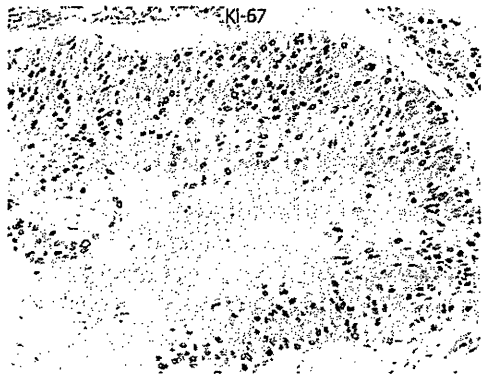


Figure 1 Immunohistochemical staining of a primary tumor sample of human esophageal squamous cell carcinomas with a Ki-67 antibody. The expression of Ki-67 was clearly identified in the nucleus of ESCCs (Magnification × 200).

bated overnight at 4 ℃ with anti-NKCC1 or anti-Ki-67 antibody. The avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector laboratories, Burlingame, CA) was visualized with diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin, dehydrated with a graded series of alcohols, cleared in xylene, and mounted.

Immunohistochemical samples stained with NKCC1 were graded semi-quantitatively by considering both the staining intensity and the percentage of positive tumor cells using an immunoreactive score (IRS)[13]. Staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining). The proportion of positive tumor cells was scored as 1 (1%-10%), 2 (11%-50%), 3 (51%-80%), or 4 (81% or more). Each sample's score was calculated as the maximum multiplied product of the intensity and proportion scores. Scores of 6 or more and scores of less than 6 were defined as high grade and low grade NKCC1 expression, respectively.

Tumor cells with nuclei containing brown immunoreactive products were considered Ki-67 positive (Figure 1). To evaluate the positive staining rate, the number of Ki-67 labeled cells was quantified in five randomly selected fields at a magnification of × 400. The positive staining rate in each case was calculated as the number of positive cells divided by the total number of examined cells in all examined fields. The mean Ki-67 labeling index was 29.4% (range, 2.9%-55.9%) in 68 primary tumor samples.

Western blotting

Cells were harvested in M-PER lysis buffer (Pierce, Rockford, IL) supplemented with protease inhibitors (Pierce, Rockford, IL). The protein concentration was measured with a modified Bradford assay (Bio-Rad, Hercules, CA). Cell lysates containing equal amounts of total protein were separated by SDS-PAGE and then transferred onto PVDF membranes (GE Healthcare, Piscataway, NJ). These membranes were then probed with the indicated antibodies, and proteins were detected using an ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ).

Small interfering RNA transfection

Cells were transfected with 10 nmol/L NKCC1 Small interfering RNA (siRNA) (Stealth RNAi™ siRNA No.HSS109914; Invitrogen, Carlsbad, CA) using the Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacturer's instructions. The medium containing siRNA was replaced with fresh medium after 24 h. The control siRNA provided (Stealth RNAi™ siRNA Negative Control; Invitrogen) was used as a negative control.

Cell cycle analysis

The cell cycle phase was evaluated 48 h after siRNA transfection by fluorescence-activated cell scoring (FACS). Briefly, cells were treated with Triton X-100 and RNase, and nuclei were stained with propidium iodide (PI) prior to DNA content measurement using a Becton Dickinson FACS Calibur instrument (Becton Dickinson, Mountain view, CA). At least 10000 cells were analyzed, and ModFit LT software (Verity Software House, Topsham, ME) was used to analyze cell cycle distribution.

Cell proliferation

Cells were seeded in 6-well plates at a density of 1.0×10^5 cells per well and incubated at 37 ℃ with 5% CO_2. siRNA was transfected 24 h after the cells seeded. Cells were detached from the flasks with trypsin-EDTA 72 h after siRNA transfection and were counted using a hemocytometer.

Real time reverse transcription-polymerase chain reaction

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). Messenger RNA (mRNA) expression was measured by quantitative real-time PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA) with TaqMan Gene Expression Assays (Applied Biosystems), according to the manufacturer's instructions. Expression levels were measured for the following genes: NKCC1 (Hs00169032_m1), MAD2L1 (Hs01554513_g1), DTL (Hs00978565_m1), BLM (Mm00476150_m1), CDC20 (Hs00426680_mH), BRCA1 (Hs01556193_m1), and E2F5 (Hs00231092_m1) (Applied Biosystems). Expression was normalized for each gene to the housekeeping gene beta-actin (ACTB, Hs01060665_g1; Applied Biosystems). Assays were performed in triplicate.

Microarray sample preparation and hybridization

Total RNA was extracted using an RNeasy kit (Qiagen). RNA quality was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Cyanine-3 (Cy3)-labeled cRNA was prepared from 0.1 μg of total RNA using a Low Input Quick Amp Labeling Kit (Agilent), according to the manufacturer's instructions. Samples were purified using RNeasy columns (Qiagen). A total of 0.60 μg of Cy3-labelled cRNA was

Table 1 Correlations between clinicopathological parameters and $Na^+/K^+/2Cl^-$ cotransporter 1 expression

Variable		NKCC1 expression		*P* value
		Low grade	High grade	
Age (yr)	< 60	12	10	0.1874
	≥ 60	16	30	
Gender	Male	25	32	0.5049
	Female	3	8	
Location of tumor	Ce/Ut	4	3	0.4346
	Mt/Lt/Ae	24	37	
Tumor size (mm)	< 50	18	30	0.4206
	≥ 50	10	10	
Histological type	Differentiated type SCC	25	21	0.0015[a]
	Poorly differentiated type SCC	3	19	
pT	pT1	10	21	0.2191
	pT2-3	18	19	
pN	negative	13	20	0.8095
	positive	15	20	
pStage	I	6	16	0.1231
	II-III	22	24	
Ki-67 labeling index		28.7 ± 2.3	29.9 ± 2.0	0.6834

Ce: Cervical esophagus; Ut: Upper thoracic esophagus; Mt: Middle thoracic esophagus; Lt: Lower thoracic esophagus; Ae: Abdominal esophagus; pT: Pathological T stage; pN: Pathological N stage; pStage: Pathological stage; SCC: Squamous cell carcinoma; [a]$P < 0.05$ *vs* control, Fisher's exact test.

fragmented and hybridized to an Agilent SurePrint G3 Human Gene Expression 8 × 60K Microarray for 17 h. Slides were washed and scanned immediately on an Agilent DNA Microarray Scanner (G2565CA) using the one color scan setting for 8 × 60K array slides.

Processing of microarray data

Scanned images were analyzed with Feature Extraction Software 10.10 (Agilent) using default parameters to obtain background-subtracted and spatially detrended Processed Signal intensities. Signal transduction networks were analyzed with Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA).

Statistical analysis

Fisher's exact test was used to evaluate the differences between proportions, and Student's *t* tests (for comparisons between two groups) and Tukey-Kramer HSD tests (for multiple comparisons) were used to evaluate continuous variables. Survival curves were constructed by the Kaplan-Meier method, and differences in survival were examined using the log-rank test. Differences were considered significant when the relevant *P* value was < 0.05.

These analyses were performed using the statistical software JMP (version 8, SAS Institute Inc., Cary, NC). Correlation analysis was performed by creating Fit Y by X plots using JMP.

RESULTS

NKCC1 protein expression in human ESCCs

An immunohistochemical examination of non-cancerous esophageal epithelia performed with the NKCC1 antibody demonstrated that cells with NKCC1 expression were chiefly confined to the lower and middle layer of the squamous epithelium but were absent from the basal and parabasal cell layers (Figure 2A). Photographs of well differentiated, moderately differentiated, or poorly differentiated ESCC tumor samples with high or low NKCC1 expression are shown in Figure 2B. NKCC1 expression was observed in the cytoplasm of ESCC cells in all groups. NKCC1 staining scores were significantly increased as histological differentiation decreased (Figure 2C).

We divided ESCC patients into 2 groups, a low grade NKCC1 expression group with staining scores < 6, $n = 28$, and a high grade NKCC1 expression group with staining scores ≥ 6, $n = 40$, and compared their clinicopathological features. We found that the percentage of poorly differentiated SCC samples was significantly higher in the high grade group (47.5%) when compared to the low grade group (10.7%) (Table 1). No correlation was found between NKCC1 expression and any other clinicopathological parameter. No correlation was found between NKCC1 expression and the Ki-67 labeling index (Table 1). Furthermore, the 5-year survival rate did not differ between the high grade group (69.9 %) and the low grade group (63.5 %) ($P = 0.501$, the log-rank test). Subgroup analysis of pStage I patients showed that the 5-year survival rate of the high grade group (86.5%) tended to be lower than that of the low grade group (100.0 %), although no significant difference was observed ($P = 0.403$, the log-rank test). These results suggest that NKCC1 plays an important role in the differentiation of ESCC cells, although a significant prognostic impact could not be determined.

NKCC1 controls cell cycle progression in ESCC cells

We examined six ESCC cell lines, TE2, TE5, TE9 TE13, KYSE70, and KYSE170, to determine NKCC1 protein expression levels. Western blotting analysis revealed that NKCC1 was highly expressed in the KYSE170 cell line, and lower levels of expression were observed in the TE2 and TE5 cell lines (Figure 3A). We conducted knockdown experiments using NKCC1 siRNA in KYSE170 cells and analyzed the effects of NKCC1 depletion on cell cycle progression. NKCC1 siRNA effectively reduced NKCC1 protein levels (Figure 3B) and NKCC1 mRNA levels (Figure 3C) in the KYSE170 cell line. The downregulation of NKCC1 induced G_2/M phase arrest in KYSE170 cells (Figure 3D). The cell counts of NKCC1 depleted cells were significantly lower when compared to those of control siRNA transfected cells 72 h after siRNA transfection (Figure 3E). Furthermore, the NKCC blocker furosemide significantly inhibited

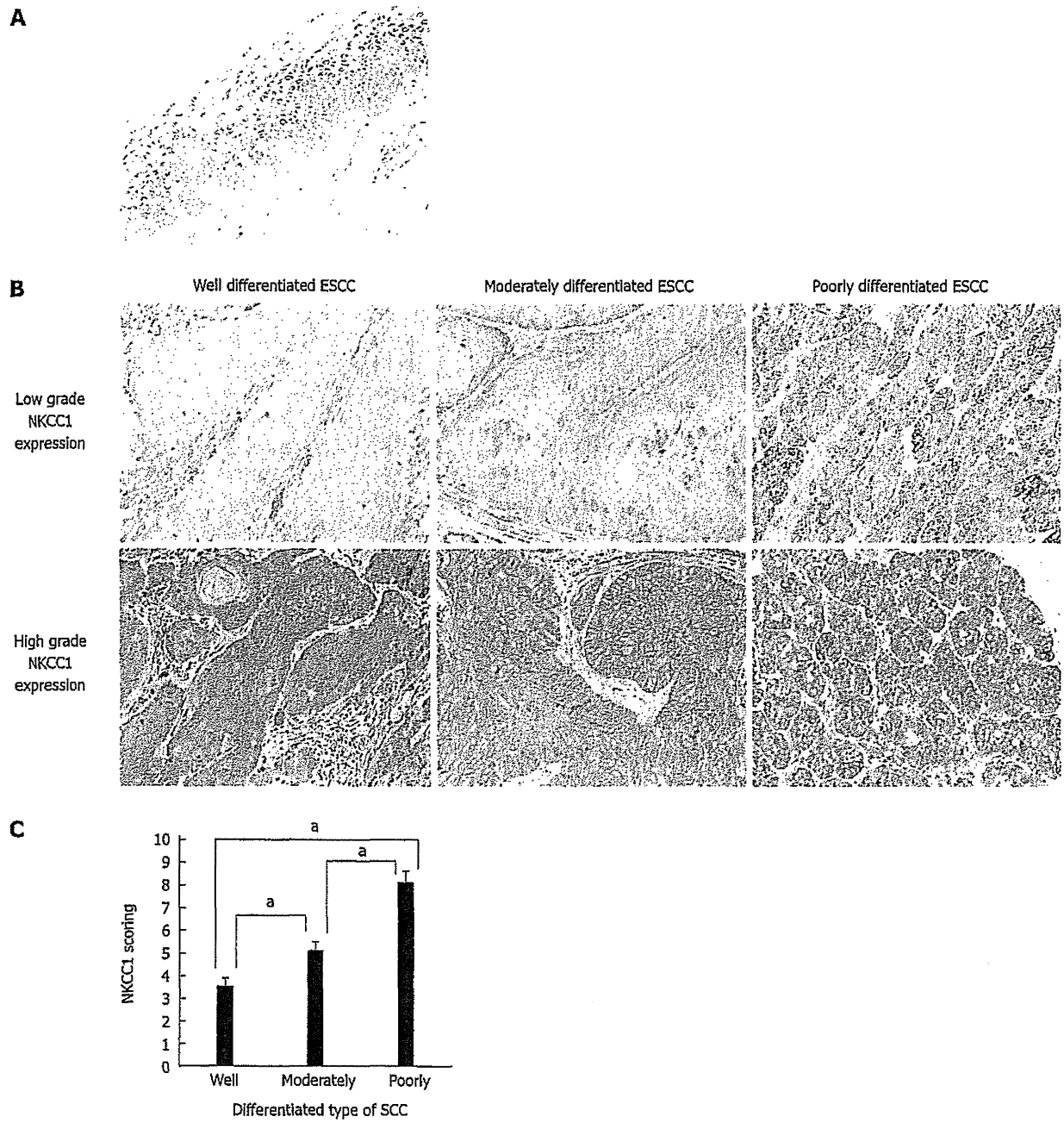


Figure 2 $Na^+/K^+/2Cl^-$ cotransporter 1 protein expression in human esophageal squamous cell carcinomas. A: Immunohistochemical staining of human esophageal epithelia with an $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) antibody. Cells with NKCC1 expression were primarily confined to the lower and middle layers of the squamous epithelium with the exception of the basal and parabasal cell layers; B: Immunohistochemical staining of well differentiated, moderately differentiated, or poorly differentiated esophageal squamous cell carcinoma (ESCC) tumor samples with high or low grade NKCC1 expression (magnification: × 200); C: NKCC1 staining scores according to the differentiation type of SCC. Mean ± SEM. Well differentiated ESCC; n = 15. Moderately differentiated ESCC; n = 31. Poorly differentiated ESCC; n = 22. $^aP < 0.05$ *vs* control, Tukey-Kramer HSD test.

the proliferation of KYSE170 cells (Figure 3F). Similar trends were found in several cell lines, including TE9, TE13 and KYSE 70, which expressed NKCC1 (Figure 4). These results suggest that NKCC1 plays an important role in regulating cell cycle progression and cell proliferation in ESCC cells.

Gene expression profiles of NKCC1 depleted cells

We analyzed the gene expression profiles of NKCC1 depleted KYSE170 cells in microarray and bioinformatics studies. Microarray analysis showed that the expression levels of 2527 genes displayed fold changes of > 2.0 in KYSE170 cells upon depletion of NKCC1. Of these genes, 1157 were upregulated and 1370 were downregulated in NKCC1 siRNA depleted KYSE170 cells. A list of 20 genes with expression levels that were the most strongly up- or downregulated in NKCC1 depleted KYSE170 cells is shown in Table 2. NKCC1 (SLC12A2) expression

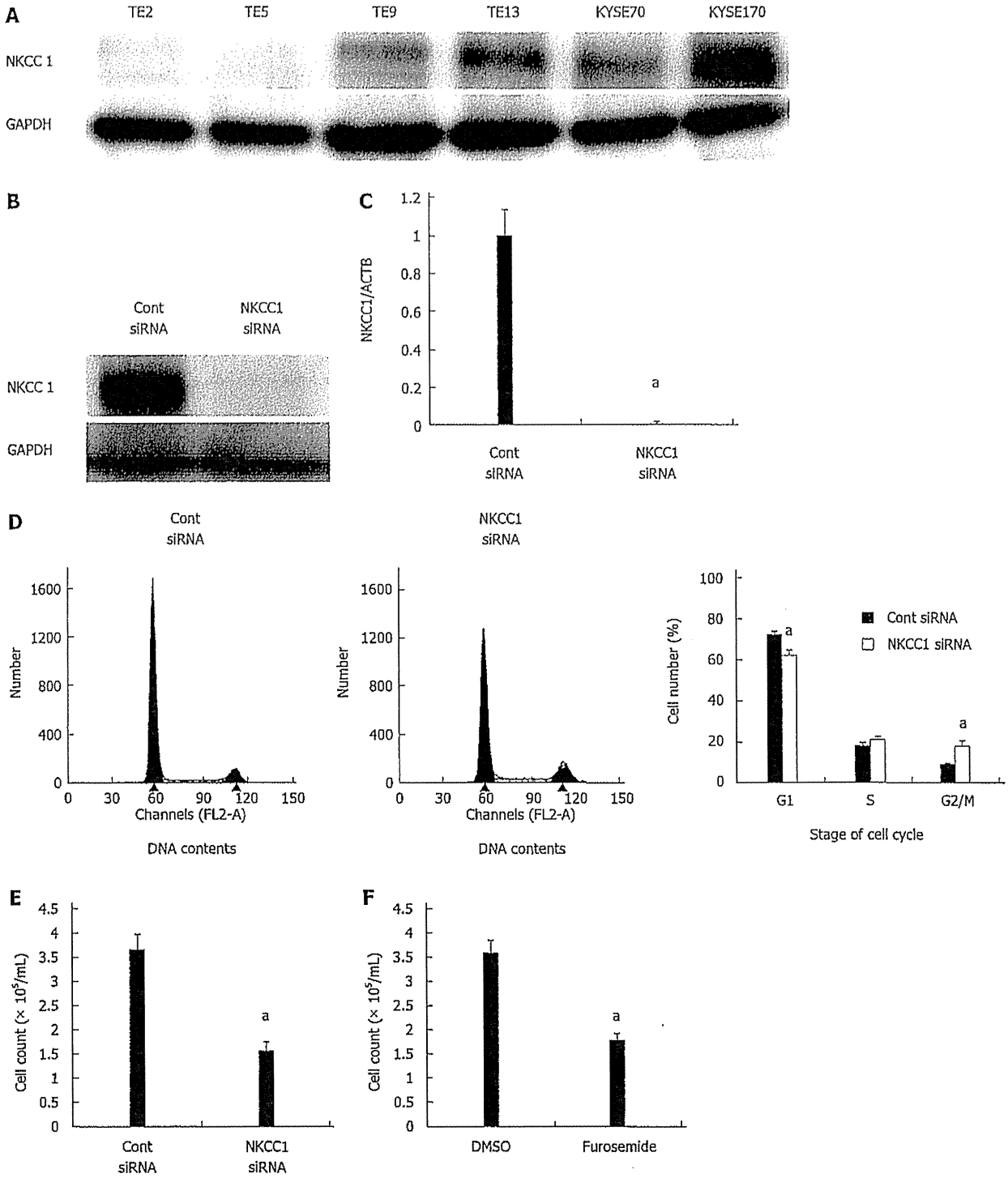


Figure 3 $Na^+/K^+/2Cl^-$ cotransporter 1 controls cell cycle progression in esophageal squamous cell carcinoma cells. A: $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) protein expression was analyzed in 6 esophageal squamous cell carcinoma (ESCC) cell lines. Western blotting revealed that NKCC1 was highly expressed in the KYSE170 cell line, and lower levels of expression were observed in TE2 and TE5 cells. B: Western blotting revealed that NKCC1 small interfering RNA (siRNA) effectively reduced the protein levels of NKCC1 in KYSE170 cells; C: NKCC1 siRNA effectively reduced the mRNA levels of NKCC1 in KYSE170 cells. The mean ± SEM. $n = 4$. $^aP < 0.05$ *vs* the control siRNA group; D: The depletion of NKCC1 induced G_2/M phase arrest in KYSE170 cells. Cells transfected with control or NKCC1 siRNA were stained with propidium iodide (PI) and analyzed by flow cytometry. The mean ± SEM. $n = 5$. $^aP < 0.05$ *vs* control siRNA; E: The depletion of NKCC1 inhibited the proliferation of KYSE170 cells. Cell number was counted 72 h after siRNA transfection. The mean ± SEM. $n = 5$. $^aP < 0.05$ (significantly different from control siRNA); F: The NKCC blocker furosemide inhibited the proliferation of KYSE170 cells. Cell number was counted 72 h after drug stimulation (500 μmol/L furosemide). The mean ± SEM. $n = 5$. $^aP < 0.05$ *vs* control (significantly different from 500 μmol/L DMSO). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

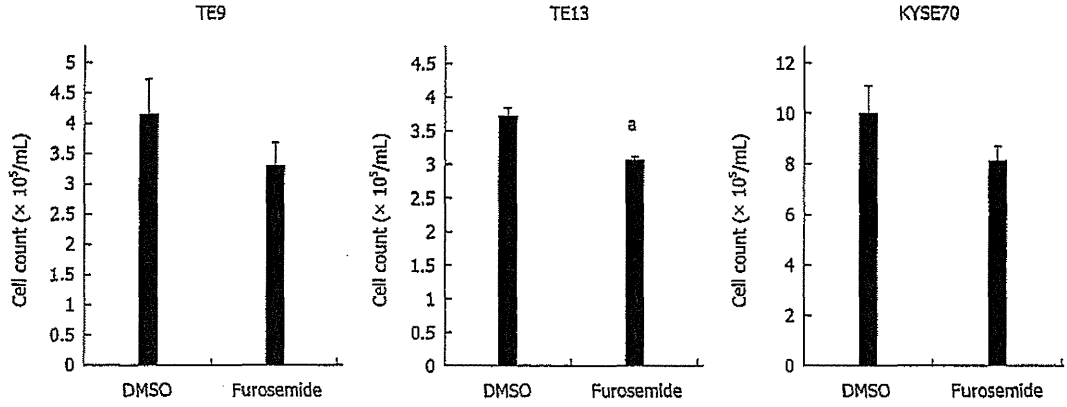
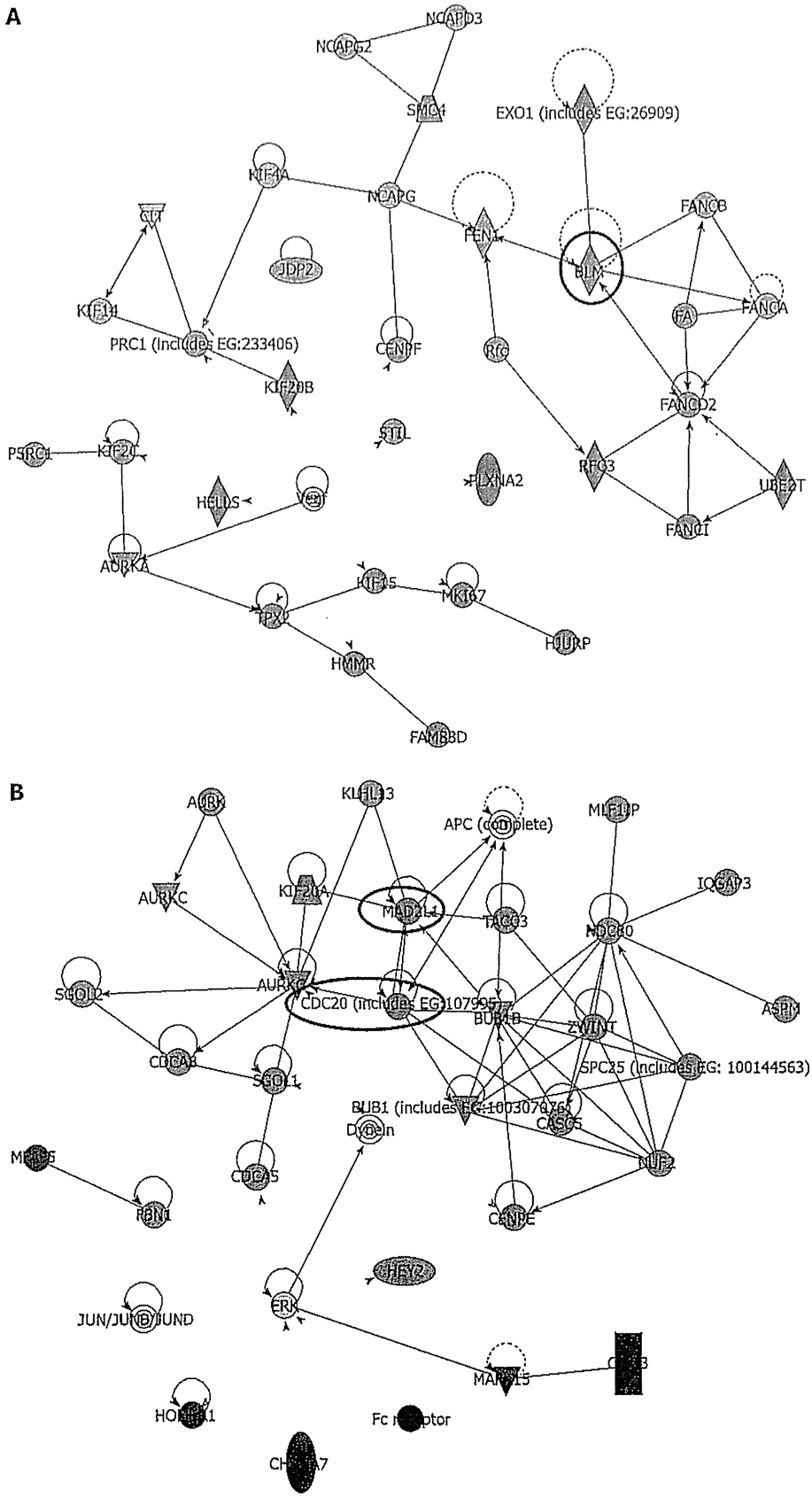


Figure 4 Effects of the $Na^+/K^+/2Cl^-$ cotransporter blocker furosemide on the proliferation of TE9, TE13 and KYSE70 cells. Cell number was counted 72 h after drug stimulation (500 μmol/L furosemide). The mean ± SEM. $n = 3$. $^aP < 0.05$ *vs* control (significantly different from 500 μmol/L DMSO).

Table 2 Twenty genes displaying the highest change in expression levels in $Na^+/K^+/2Cl^-$ cotransporter 1 depleted KYSE-170 cells

Gene Symbol	Gene ID	Gene Name	Fold Change
Upregulated Genes			
C18orf34	NM_001105528	Chromosome 18 open reading frame 34	155.49
KCNA6	NM_002235	Potassium voltage-gated channel, shaker-related subfamily, member 6	140.4
CCDC147	NM_001008723	Coiled-coil domain containing 147	105.98
C20orf202	NM_001009612	Chromosome 20 open reading frame 202	86.17
A1CF	NM_138933	APOBEC1 complementation factor	70.98
SH3GL2	NM_003026	SH3-domain GRB2-like 2	70.93
PTGFR	NM_001039585	Prostaglandin F receptor (FP)	66.99
NDN	NM_002487	Necdin homolog (mouse)	66.45
INPP5D	NM_001017915	Inositol polyphosphate-5-phosphatase, 145 kDa	52.83
CYP2E1	NM_000773	Cytochrome P450, family 2, subfamily E, polypeptide 1	52.44
AGBL3	NM_178563	ATP/GTP binding protein-like 3	50.88
UBTFL1	NM_001143975	Upstream binding transcription factor, RNA polymerase I -like 1	47.88
PADI2	NM_007365	Peptidyl arginine deiminase, type II	46.83
CCR1	NM_001295	Chemokine (C-C motif) receptor 1	44.86
ARC	NM_015193	Activity-regulated cytoskeleton-associated protein	44.41
COLEC10	NM_006438	Homo sapiens collectin sub-family member 10 (C-type lectin)	44.28
DNAH6	NM_001370	Dynein, axonemal, heavy chain 6	41.96
BOLL	NM_033030	Bol, boule-like (Drosophila)	41.31
CORO2B	NM_006091	Coronin, actin binding protein, 2B	41.04
MUC7	NM_152291	Mucin 7, secreted	36.97
Downregulated Genes			
NPFFR1	NM_022146	Neuropeptide FF receptor 1	-54.97
LRRFIP1	NM_001137550	Leucine rich repeat (in FLII) interacting protein 1	-44.72
PPIL6	NM_173672	Peptidylprolyl isomerase (cyclophilin)-like 6	-44.46
CRHR2	NM_001883	Corticotropin releasing hormone receptor 2	-39.78
CMTM2	NM_144673	CKLF-like MARVEL transmembrane domain containing 2	-39.62
C5	NM_001735	Complement component 5	-39.13
KCNMA1	NM_001014797	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	-38.59
HESX1	NM_003865	HESX homeobox 1	-33.03
SLC22A2	NM_003058	Solute carrier family 22 (organic cation transporter), member 2	-32.49
WNT8B	NM_003393	Wingless-type MMTV integration site family, member 8B	-32.17
GRIA1	NM_000827	Glutamate receptor, ionotropic, AMPA 1	-31.27
ZNF367	NM_153695	Zinc finger protein 367	-30.04
GPR128	NM_032787	G protein-coupled receptor 128	-29.88
SLC12A2	NM_001046	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	-28.92
KCNG2	NM_012283	Potassium voltage-gated channel, subfamily G, member 2	-28.3
ECT2L	NM_001077706	Epithelial cell transforming sequence 2 oncogene-like	-27
ERMN	NM_020711	Ermin, ERM-like protein	-26.61
DPP10	NM_020868	Dipeptidyl-peptidase 10 (non-functional)	-26.58
TSPAN7	NM_004615	Tetraspanin 7	-25.54
APOA1	NM_000039	Apolipoprotein A-I	-25.21


A
NCAPD3
NCAPG2
SMC4
EXO1 (includes EG:26909)
NIF4A
NCAPG
CIT
PEN1
FANCB
JDP2
BLM
KIF11
FA
FANCA
PRC1 (includes EG:233406)
CENPF
RFC
KIF20B
FANCD2
PSRC1
KIF2C
STIL
PLXNA2
RFC3
UBE2T
HELLS
FANCI
AURKA
KIF15
MKI67
TPX2
HJURP
HMMR
FAM83D
B
AURKB
KLHL13
APC (complete)
MLF1IP
AURKC
KIF20A
MAD2L1
TACC3
IQGAP3
NDC80
SGOL2
AURKC
CDC20 (includes EG:1079957)
BUB1B
ZWINT
ASPM
CDCA8
SGOL1
SPC25 (includes EG: 100144563)
BUB1 (includes EG:100307065)
CASC5
Dynein
NUF2
CDCA5
CENPE
HER2
ERK
JUN/JUNB/JUND
MAPK15
Fc receptor