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Review Article

The emerging role of KCl cotransport in tumor biology

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Abstract: The electroneutral KCl cotransport carried out by the KCl cotransporter family (KCC) plays a significant role in the ionic and osmotic homeostasis of epithelial cells. Here we review the emerging importance of KCl cotransport in epithelial carcinogenesis and tumor malignant behaviors. The malignant transformation of cervical epithelial cells is associated with the differential expression of volume-sensitive KCC isoforms. The loss-of-function KCC mutant cervical cancer cells exhibit inhibited cell growth accompanied by decreased activities of the cell cycle regulators and matrix metalloproteinase. Additionally, insulin-like growth factor-1 (IGF-1) stimulation of KCl cotransport plays an important role in IGF-1 signaling to promote growth and spread of gynecological cancers. IGF-1 upregulates KCC3 and KCC4 which are differentially required for cancer cell proliferation and invasiveness. KCC3 overexpression downregulates E-cadherin/β-catenin complex formation by inhibiting the transcription of *E-cadherin* gene and accelerating the proteosome-dependent degradation of β-catenin protein. That therefore promotes the epithelial-mesenchymal transition of cervical cancer cells, and thereby stimulating tumor progression. Moreover, epidermal-growth factor (EGF) and IGF-1 stimulate the membrane recruitment of KCC4 at lamellipodia through myosin Va-actin trafficking route. KCC4 functions as a membrane scaffold for the assembly of signal complexes via the association with the actin-binding protein, ezrin. The molecular studies of surgical specimens suggest that the expression of KCC3, KCC4, and their stimulators, EGF or IGF-1, exhibit a close association with the clinical outcome of cancer patients. Therefore, KCC3, KCC4, EGF, and IGF-1 may be a panel of biomarkers to predict cancer patient outcome.

Keywords: KCl cotransport, proliferation, migration, metastasis, cervical cancer, ovarian cancer

Introduction of KCl cotransport

As originally described in red blood cells (RBCs), KCl cotransport is the electroneutral symport of K^+ and Cl^- across the plasma membrane that can be activated by cell swelling [1, 2] and sulfhydryl-alkylating reagent N-ethylmaleimide [3, 4]. KCl cotransport is necessary for the cell volume regulation, transepithelial ion transport, and maintenance of intracellular Cl^- concentration [4]. The KCl cotransport activity is mediated by the four homologous *SLC12* gene family of electroneutral cation-chloride cotransporters, namely KCC1 [5], KCC2 [6], KCC3 [7-9], and KCC4 [8] (*SLC12A4-7*, respectively). The four KCC isoforms are 65-75% identical in protein sequences. The phylogenetic analyses revealed that four KCC isoforms fall into two subgroups that KCC1 pairs with KCC3 whereas KCC2 pairs with KCC4 [8]. The four KCC isoforms share a common protein structure with a central core of twelve hydrophobic transmembrane domains flanked by two hydrophilic N- and C-terminal intracellular domains [8]. A large extracellular loop between 5th and 6th transmembrane domains that conserves three identical N-linked glycosylation sites has also been predicted from the published sequences of four KCC isoforms [8]. However, these transporters differ in amino acid residues within key transmembrane domains and in the distribution of putative phosphorylation sites within the N- and C-terminal cytoplasmic domains.

KCl cotransport plays a significant role in ionic and osmotic homeostasis of RBC and epithelial cells [4, 10]. The activities of KCC1, KCC3, and KCC4 are osmotically sensitive and participate

in the cell volume regulation [11]. The neuronal-specific isoform KCC2 exhibits the constitutive KCl cotransport activity under isotonic conditions and is therefore important for the maintenance of intraneuronal Cl^- concentration [12, 13]. Regulation of KCl cotransport is likely complicated; however studies in RBCs have identified several regulators of KCl cotransport activity [4, 14]. Under physiological conditions, KCl cotransport activity can be stimulated by cell swelling, low intracellular pH, high partial pressure of oxygen, and urea [14]. Pharmacological activators include the thiol-alkylating reagent N-ethylmaleimide (NEM) and the oxidizing reagents (e.g. H_2O_2, hydroxylamine, and NO). The intracellular signaling cascades of phosphorylation/dephosphorylation events also play important roles in regulating KCl cotransport activity [15, 16]. KCl cotransport is stimulated by kinase inhibition (e.g. thiol reagents, staurosporine, and genistein) and inactivated by phosphatase inhibition (e.g. okadaic acid and calyculin) [10, 15, 16]. Protein phosphatase PP1A and PP2A and serine-threonine kinase WNK family are implicated in modulating the dephosphorylation and phosphorylation of KCC isoforms, respectively [17, 18]. Furthermore, a recent study based on the biochemical analyses of KCC3 polypeptides has identified two key phosphorylation sites in the intracellular C-terminal tail [19]. These two phosphorylation sites are conserved among four KCC isoforms and are critical for regulating KCl cotransport activity. Surface expression of KCC3 displays high phosphorylation levels of these two sites under isotonic conditions in which KCl cotransport activity is low. In contrast, these two sites are rapidly dephosphorylated in response to hypotonic stress in parallel with increased KCl cotransport activity.

Despite RBC has been the well-studied model for understanding the cellular physiology of KCl cotransport, most functional studies of KCC were done on KCl cotransport fluxes without knowing the physiological and molecular details of individual KCC isoforms. Knockout mouse models and human diseases which link to the mutation of specific KCC genes have greatly advanced our knowledge of the physiological functions and pathological defects of individual KCC isoforms. The molecular characterization, physiological functions, and pathological defects of KCC isoforms are described as follows.

KCC1 (SLC12A4)

KCC1, along with KCC2, are two first KCC isoforms which were cloned and identified [5]. The human *KCC1* gene is on chromosome 16q22 and encodes for a protein product of 1085 amino acids that has highest identity (75%) with KCC3 among three isoforms. KCC1, ubiquitously detected in mammalian cells and tissues analyzed by Northern blot analyses, is considered as the "housekeeping" transport mechanism in cell volume regulation and transepithelial ion transport [20, 21]. The structure-function relationship study on mammalian KCC1 revealed that both the C-terminal and the membrane-proximate region of N-terminal domain are necessary for KCl cotransport function [22]. The removal of the N-terminal 117 amino acids from KCC1 produced a dominant-negative loss-of-function phenotype for the function of all four KCC isofroms in many types of cells [22-24].

Despite human KCC1 is expressed in every cell and tissue tested so far, the specific cellular and physiological function of KCC1 have remained unclear due to the absence of a KCC1-associated human disease. Pathological activation of KCl cotransport in RBC is thought to contribute to the dehydration and deformation of sickle blood cells and is implicated in the sickle cell disease [25-27]. KCC1 has therefore been proposed as a modifier gene in sickle cell disease [21]. A recent study has shown that the KCl cotransport activity in RBCs is undiminished in *KCC1* knockout mice, suggesting other unknown functions of KCC1 in RBCs [28].

KCC2 (SLC12A5)

The human KCC2 gene is on chromosome 20q13 and encodes for a protein product of 1116 amino acids [6, 12, 13]. KCC2, a neuronal specific KCC isoform, is unique in mediating KCl cotransport constitutively under isotonic conditions; whereas the other three KCC isoforms are only active in response to the cell swelling by hypotonic stress. KCC2 displays a distinct C-terminal insertion rich in prolines and negatively charged amino acids that confers the constitutive isotonic activity [29, 30]. This region encompasses two predicted PEST [Proline/E (glutamate)/Serine/Threonine] sequences that are completely unique to KCC2. Depending on the chemical concentration gradients of K^+ and Cl^-, this transporter can operate as a net

efflux or influx pathway [6, 12].

The expression of KCC2 is detected in the developing synapse and in adult neurons throughout the central nervous system, especially in neurons of the cortex, hippocampus, and the cerebellar granular cell layer [6]. The physiological function of KCC2 in the mature brain is to lower the intraneuronal Cl^- concentration below its electrochemical equilibrium and thereby facilitating the maturation of inhibitory γ-aminobutyric acid responses. It is crucial in promoting the synaptic inhibition, controlling the central nervous system excitability, and inhibiting the postsynaptic potential. Moreover, several genetic and functional evidences have shown that KCC2 is necessary for the development of central nervous system. Complete deletion of *KCC2* gene in mice causes neonatal death due to the severe motor deficit that also abolishes the respiration [31]. Mice with partial deletion of KCC2 die from general seizure and respiratory failure by the postnatal stage [32]. Furthermore, adult heterozygous mice show elevated susceptibility for epileptic seizure and increased resistance to the anticonvulsant drug, suggesting the involvement in the pathology of human epilepsy.

In addition to the developmental role in the inhibitory synapses, KCC2 is crucial for the maturation of dendritic spines and functional excitatory synapses [33]. Evidences form electrophysiological and immunefluorescent studies have revealed that neurons lacking KCC2 expression develop immature filopodia-like dendritic protrusions in parallel with a reduction in active synapses. Moreover, the morphogenic role of KCC2 in dendrite maturation is mediated by the specific structural interaction between KCC2 with the actin-binding ezrin/radixin/moesin protein, 4.1N, rather than the KCl cotransport activity of KCC2.

KCC3 (SLC12A6)

The human *KCC3* gene is located on chromosome 15q13 and is colocalized with the gene for myoclonal epilepsy [8, 9]. The mRNA transcripts of KCC3 are abundant in muscle, brain, spinal cord, kidney, heart, pancreas, and placenta [8, 9]. Two main 5'-splice variants of KCC3, KCC3a and KCC3b, have been identified [33]. The two KCC3 variants have different N-terminal ends due to the deletion at exon 1 in the shorter KCC3b. Both KCC3 variants contain 5 potential N-linked glycosylation sites in the extracellular loop between the 5th and 6th transmembrane domain and share about 77% amino -acid identity with KCC1 and 73% with KCC2. The predicted KCC3a protein is longer by 50 amino acids and encompasses several unique phosphorylation sites that are absent in the N-terminus of KCC3b. The distribution of KCC3a is more widespread than that of KCC3b and is particularly abundant in the kidney [35].

Little is known about the cellular and physiological properties of KCC3 proteins. The expression of KCC3 is correlated with the myelinization in the rodent central nervous system, suggesting a role in the physiology of myelinization [35]. KCC3 also plays an important role in regulating cell growth and proliferation [23, 36, 37]. Moreover, the functional defects of *KCC3* knockout mice have provided clues about the physiological functions of KCC3. KCl cotransport activity in RBCs is decreased in *KCC3* knockout mice, and almost completely abolished in mice lacking KCC1 and KCC3, indicating that KCl cotransport activity of mouse RBC is largely mediated by KCC3 [28]. Loss of KCC3 also causes arterial hypertension, slowly progressive deafness, peripheral neurodegeneration and reduced seizure threshold in mice [34, 38, 39]. Furthermore, several genetic and functional evidences have shown that loss-of-function mutations in human *KCC3* gene cause a rare autosomal disease, the agenesis of the corpus callosum with peripheral neuropathy (ACCPN) [34]. The syndrome of ACCPN includes the severe sensorimotor neuropathy associated with mental retardation, dysmorphic features, and complete or partial agenesis of the corpus callosum. Indeed, the neurodegenerative phenotypes of *KCC3* knockout mice, including locomotor deficit, peripheral neuropathy, and the sensorimotor gating deficit, are consistent with certain features of ACCPN. However, the pathophysiological properties of KCC3 underlying this neurological deficit is not yet clear. The axonal and periaxonal swelling proceeding the peripheral neurodegeneration of *KCC3* knockout mice implicates that KCC3-mdiated cell volume regulation is critical for the development and maintenance of peripheral axon and myelin [40].

KCC4 (SLC12A7)

The human *KCC4* gene is located on chromo-

Table 1. The physiological functions and pathological defects of KCC isoforms

Human gene name	Protein name	Human gene locus	Tissue distribution	Physiological functions	Potential human disease(s)	Functional defects
SLC12A4	KCC1	16q22	Ubiquitously expressed	·Cell volume regulation	·Sickle cell anemia? (modifier gene)	·Dominant-negative effect on KCC activity
SLC12A5	KCC2	20q13	Neurons throughout the central nervous system	·Cell volume regulation ·Maturation of GABA response in CNS	·Epilepsy?	·Neonatal death (Null) ·General seizure and respiratory failure (partial null)
SLC12A6	KCC3	15q14	Muscle, brain, placenta, kidney, heart and pancreas	·Cell volume regulation ·Cell proliferation	·ACCPN ·Sickle cell anemia?	·Deafness ·Peripheral neurodegeneration ·Hypertension
SLC12A7	KCC4	5p15	Widely distributed, robustly in kidney and heart	·Cell volume regulation ·K^+ transport	·Renal tubule acidosis?	·Renal tubule acidosis ·Deafness

ACCPN, agenesis of the corpus callosum with peripheral neuropathy.

some 5p15 and encodes for a protein product of 1083 amino acids which shares high identity (about 75%) with KCC2 [8]. However, unlike KCC2, KCC4 does not mediate the constitutive KCl cotransport under isotonic conditions but the swelling-activated cotransport [11]. Like KCC1, the mRNA transcripts of KCC4 are widely expressed in various tissues. The protein expression of KCC4 is particularly robust in the kidney and is localized at the basolateral membrane of type-A intercalated cells and proximal tubules [18, 41]. It was therefore postulated that the K^+ and Cl^- efflux-mediated by KCC4 play an important role in the salt absorption of the distal convoluted tubule [41]. KCC4 may have some undetermined functions in the central nervous system as the significant expression of KCC4 has also been demonstrated in cranial nerves, spinal cord and peripheral nerves [42]. Moreover, mice with *KCC4* gene disruption exhibit deafness and renal tubular acidosis [43]. The renal tubular acidosis phenotype may result from the defect in the acid secretion of intercalated cells. The impaired K^+ recycling from outer hair cells into supporting Deiter's cells may lead to the degeneration of outer hair cells of the cochlear and thereby the deafness. The physiological functions and pathological defects of four KCC isoforms are summarized in **Table 1**.

KCl cotransport activity affects the tumor invasion and proliferation

The emerging importance of KCl cotransport in the epithelial carcinogenesis and tumor malignant behaviors has been established from several studies based on the models of gynecological cancers over the past decade. KCl cotransport is one of the major K^+ and Cl^- flux pathways in epithelial cells and plays a significant role in the ionic and osmotic homeostasis [4, 10]. Homeostasis of cell volume is a fundamental property of mammalian cells. Cell proliferation and migration are accompanied by the dynamics in cell shape and volume. The metabolism, mitosis, proliferation, and migration of cancer cells are usually more active than those of normal cells. The close linkage between cell volume regulation, cell proliferation, and cell migration leads to an obvious question that is "Do cancer cells develop an advantage in cell volume regulation that benefits them in tumor malignant behaviors?"

It was first tested if the malignant transformation of human epithelial cells is associated with the differential expression of volume-sensitive KCC isoforms. The molecular identification and functional flux studies demonstrated that the malignant transformation of cervical epithelial cells is accompanied by the differential expression of volume-sensitive KCl cotranposrt activities [44]. Cervical carcinogenesis is accompanied by the up-regulation of mRNA transcripts of KCC1, KCC3 and KCC4. Moreover, the growth and invasion of cervical cancer cells are strongly correlated with the expression and activity of KCC [23]. Functional assays of KCl cotransport

activation by osmotic swelling, staurosporine, and NEM in human cervical cancer cells indicate that the removal of N-terminal 117 amino acids from KCC1 produces a dominant-negative loss-of-function phenotype for KCl cotransport and thereby elevating intracellular Cl^- concentration. Alteration of intracellular Cl^- concentration affects the activity of retinoblastoma (Rb) protein and cdc2 kinase, two key cell cycle regulators controlling the progression from G_1 into S phase and from G_2 into M phase, respectively [36]. The loss-of-function KCC mutant cervical cancer cells exhibit inhibited cell growth accompanied by decreased activity of Rb protein and cdc2 kinase. Reduced cellular invasiveness is in parallel by decreased expression of $\alpha_v\beta_3$ and $\alpha_6\beta_4$ integrins, accompanied by inhibited activity of matrix metalloproteinase (MMP)-2 and -9. Inhibition of tumor growth in severe combined immunodeficient mice confirms the critical role of KCl cotransport in promoting cervical cancer growth and invasion. Taken together, KCl cotransport plays a crucial role in the proliferation and invasion of cancer cells as well as in the epithelial carcinogenesis. The mechanisms by which KCl cotransport affects the tumor invasion and proliferation are summarized in **Figure 1**.

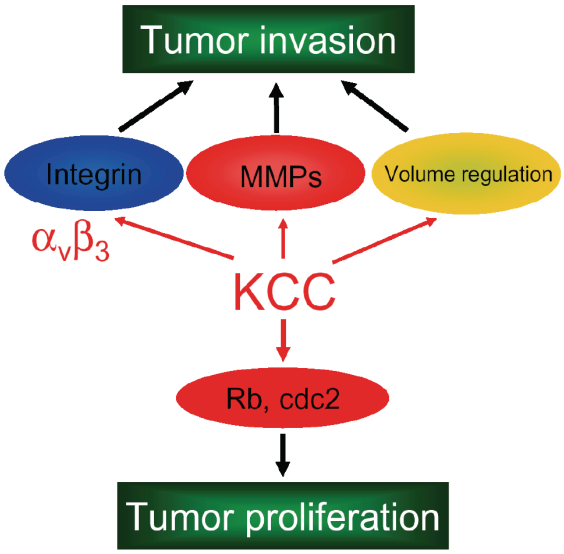

Figure 1. Schematic diagram indicating the mechanisms by which KCl cotransport activity affects the tumor invasion and proliferation. KCl cotransport activity is involved in cell volume regulation, matrix metalloprotease (MMP) activity, and integrin activation, thereby affecting tumor cell invasion. The tumor cell proliferation is affected by KCl cotransport activity through modulating the phosphorylation of cell cycle gene products retinoblastoma (Rb) and cdc2 kinase, two key regulators controlling cell cycle progression.

The cooperation between KCl cotransport and certain growth factors has also been implicated in modulating the proliferation and invasive migration of cancer cells. Overexpression of specific growth factors significantly enhances the invasive and metastatic properties of cancer cells, which poses serious problems to the successful treatment of neoplastic disease. Insulin-like growth factor 1 (IGF-1) has been implicated in promoting malignant phenotypes, such as mitogenesis, invasiveness, metastasis and anti-apoptosis, in most types of cancer [45, 46]. The good correlation between the expression level of IGF-1 and that of KCC polypeptides in the surgical specimens of cervical cancers, ovarian cancers, and breast cancers suggest a likely autocrine or paracrine regulation of IGF-1 on KCl cotransport activity and expression *in vivo* [47, 48]. Studies in the cell line models indicate that IGF-1 increases the expression and activity of KCl cotransport through the differentially activation of the downstream phosphophatidylinostiol 3-kinase (PI3K) and extracellular signal –regulated kinase-1/2 (Erk1/2) MAP kinase signaling pathways. Pharmacological inhibition and genetic modification of KCl cotransport activity demonstrate that KCl cotranpsort is necessary for IGF-1-induced cancer cell invasiveness and proliferation. Furthermore, IGF-1 upregulates KCC3 and KCC4 which are differentially required for tumor cell proliferation and invasiveness [36, 48]. In summary, the activation of KCl cotransport by IGF-1 plays an important role in IGF-1 signaling to promote growth and spread of gynecological cancers.

The important role of KCC3 in epithelial-mesenchymal transition (EMT)

Cancer development and progression is a multistep process that involves the uncontrolled growth of tumor cells, detachment from the primary tumor, migration through the extracellular matrix, invasion through the basal membrane, and ultimately cancer metastasis. The contribution of individual KCC isoforms in cancer development and progression has been addressed in recent studies [27, 48]. Real-time reverse transcription-PCR on samples collected by laser mi-

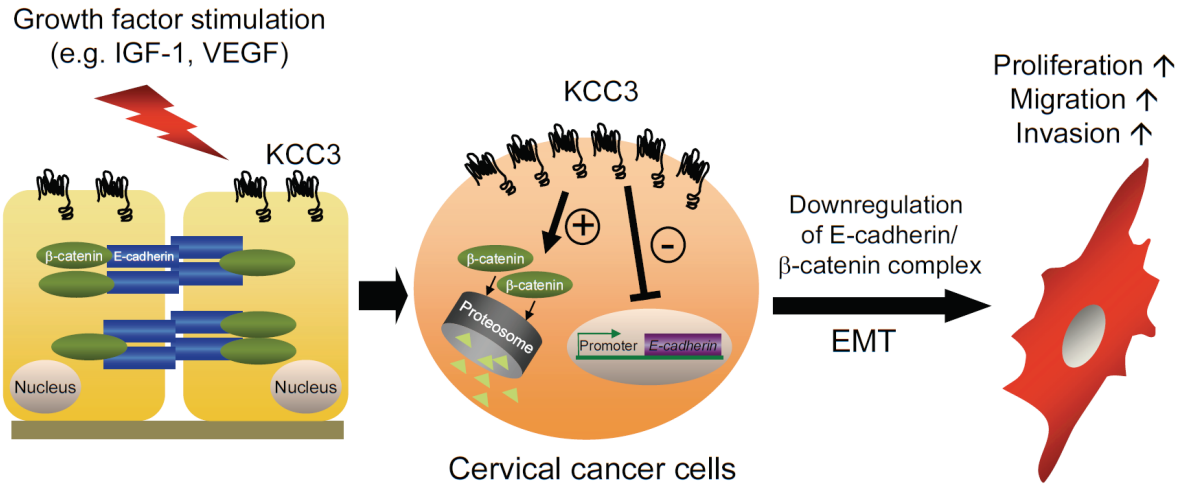

Figure 2. KCl cotransporter KCC3 downregulates E-cadherin/β-catenin complex formation to promote EMT that is important for cervical cancer cell invasiveness. The oncogenic growth factors such as IGF-1 and VEGF are known to stimulate the activity and expression of KCC3 in cervical cancer cells, ovarian cancer cells and breast cancer cells. The abundances of E-cadherin and β-catenin are affected by the expression and activity of KCC3 via the inhibition on transcription of *E-cadherin* gene and the acceleration in proteosome-dependent degradation of β-catenin protein. The disruption of E-cadherin/β-catenin complex formation causes the dysfunction of cell-cell junction system, thereby triggering cancer proliferation, invasive migration, and ultimately cancer progression. *Modified from Cancer Research 2007; 67:11064-11073 [49].*

crodissection indicates that KCC3 is the most abundant isoform in primary tumors of cervical carcinoma and ovarian cancer [24, 49]. The expression level of KCC3 is closely correlated with tumor size of cervical carcinoma, an indicator of tumor growth *in vivo*. In addition, KCC3 overexpression enhances cervical cancer proliferation *in vitro*. These are consistent with the previous study showing that KCC3 plays an important role in cell growth regulation via the modulation of cell cycle progression [36].

Overexpression of KCC3 and the consequent upregulation of KCl cotransport activity also benefit cancer cells in the epithelial-mesenchymal transition (EMT) [49], a key event occurring in cancer development and progression. Acquisition of EMT in cancer cells is associated with disrupted epithelial integrity, increased invasive migration, and cancer metastasis [50-52]. Studies in cervical cancer cell lines demonstrate that the characteristics of EMT appear in KCC3-overexpressed tumor cells, including the elongated cell shape, increased cell scattering, downregulated epithelial markers (E-cadherin and β-catenin), and upregulated mesenchymal marker (vimentin) [49]. The blockade of KCC activity in tumor cells leads to the increase in both the abundances and the association of E-cadherin and β-catenin in cell-cell junctions. Some cellular functions are enhanced by KCC3 overexpression, such as increased invasiveness and proliferation, and weakened cell-cell association. KCC3 overexpression decreases the mRNA level of E-cadherin. The promoter activity assays of various regulatory sequences reveal that KCC3 expression is a potent negative regulator for human *E-cadherin* gene expression. The proteosome inhibitor restores the decreased protein abundance of β-catenin by KCC3 overexpression. In the surgical specimens of cervical carcinoma, the decreased expressions of E-cadherin as well as the loss of cell integrity are accompanied by the increased KCC3 abundance. Meanwhile, vimentin begins to appear at the invasive front and becomes significantly expressed in the tumor nest. Taken together, KCC3 downregulates E-cadherin/β-catenin complex formation by inhibiting the transcription of *E-cadherin* gene and accelerating the proteosome-dependent degradation of β-catenin protein. The disruption of E-cadherin/β-catenin complex formation promotes EMT, thereby facilitating tumor progression (summarized in **Figure 2**). The close linkage between KCC3 abundance, cell proliferation, invasiveness, and EMT suggests that the expression and activity of

KCC3 may serve as a selective advantage for cancer cells in malignant behaviors.

The important role of KCC4 in cancer invasion and metastasis

The important role of KCC4 in tumor malignant behavior, such as cancer cell invasiveness and tumor metastasis has been identified in a recent study [24]. In surgical specimens of metastatic cervical and ovarian cancers, both mRNA transcripts and protein levels of KCC4 are the most abundant one among that of four KCC isoforms. The significant association between KCC4 abundance and prognostic factors of cervical cancer patients, such as parametrium invasion, pelvic lymph node metastasis, and the recurrence-free or overall survival rates, suggest the important role of KCC4 in cancer metastasis. Studies based on the *in vitro* cell line model indicate that cancer cell invasiveness is enhanced by KCC4 overexpreesion but attenuated by KCC4 knockdown. Furthermore, the reduced cellular invasive migration is associated with decreased MMP-2 activity. These indicate that cancer cells benefit from KCC4 overexpression in the invasive migration and possibly in the tumor metastasis.

The novel mechanisms by which specific growth factors modulate the biosynthesis and functions of KCC4, thereby promoting cancer cell invasiveness, have been highlighted. IGF-1 and EGF are known to be overexpressed in most types of cancer tissues and notably contribute to cancer invasiveness and metastasis [46, 53-55]. In the metastatic cancer tissues, KCC4 colocalizes with IGF-1 or EGF, indicating a likely *in vivo* stimulation on KCC4 functions by growth factors. Chronic stimulation with IGF-1 upregulates the *de novo* proteins synthesis of KCC4 that is important for IGF-1 signaling to promote invasive migration of gynecologic cancer cells [48]. Moreover, IGF-1 and EGF stimulate the rapid recruitment of KCC4 from a presumably inactive cytoplasmic pool, such as endoplasmic reticulum and Golgi, to the active plasma membrane target one along the actin cytoskeleton. PI3K activation is the dominant signal controlling KCC4 trafficking. Throughout the trafficking process, KCC4 is incorporated into the specialized membrane microdomain, lipid rafts, that function as a platform for the association between KCC4 and myosin Va, an actin-dependent motor protein. Like KCC2, surface expression of KCC4 exhibits a structural role in the organization of signaling complex in addition to ion transport. KCC4 and ezrin, an actin-binding protein which functions as the membrane cytoskeleton linker, colocalize at lamellipodia of migratory cancer cells. Interference with KCC activity by either a pharmacologic inhibitor or a dominant-negative loss-of-function mutant profoundly suppresses the IGF-1–induced membrane trafficking of KCC4 and the structural interaction between KCC4 and ezrin near the cell surface. Taken together, KCC4 can function as a plasma membrane scaffold protein with ezrin in the assembly of cytoskeletal reorganization complex. The complex of KCC4 and ezrin may be important for regulating the integrity of cortical cytoskeleton and maintaining cell adhesion, which are required for cellular invasive migration (illustrated in **Figure 3**).

Potential clinical implication of KCC

Despite the significant improvement in both diagnostic and therapeutic modalities for the treatment of cancer patients, tumor metastasis and cancer recurrence still represent the major cause of mortality [56, 57]. It is important to identify target genes that are consistently upregulated in metastatic and recurrent cancers as the reliable biomarker to predict occurrence, progression or prognosis of human cancers. The previous studies have highlighted the important role of KCl cotransport family in tumor development and progression and have provided the rationale for its application in predicting clinical prognosis of cancer patients. For example, KCC polypeptides and its stimulator, IGF-1 protein, are scanty in normal cervical epithelial and noncancerous stromal tissues, but are abundantly expressed in adjacent cervical cancer tissues [47, 48]. In addition, IGF-1 and KCC colocalize in the surgical specimens of breast cancer, cervical cancer and ovarian cancer, suggesting the cooperation between IGF-1 and KCC in tumor progression. Moreover, the expression of IGF-1 and KCC in surgical specimens shows good linear correlation that in turn corresponds well to tumor size, the *in vivo* indicator of tumor progression. The close association between the expression of individual KCC isoforms and clinical outcome of cancer patients has also been investigated. Real-time reverse transcription-PCR on samples collected by laser microdissection and immunofluorescent stainings with different KCC isoform anti-

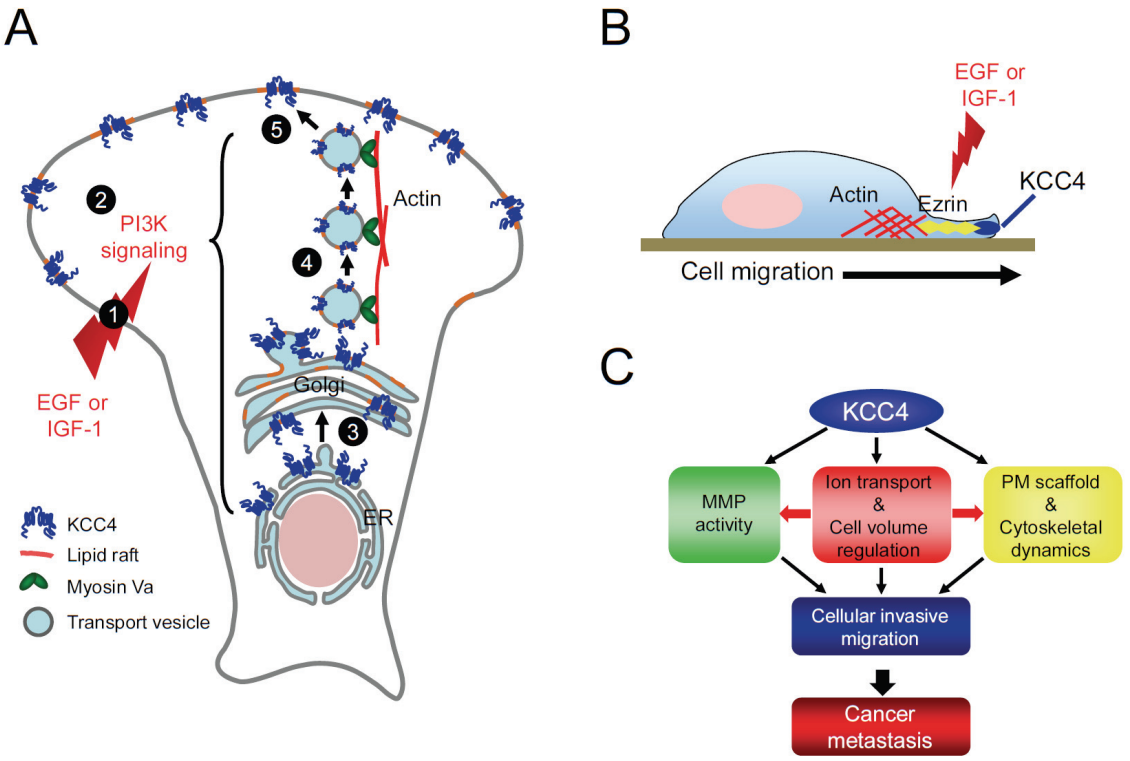

Figure 3. Motor-dependent trafficking of KCC4 is important for cancer cell invasion. (A) The recruitment of KCC4 from the inactive storage pool in the cytosol to the active membranous target pool at the lamellipodia is sensitive to IGF-1 and EGF stimulation. PI3K activation is the dominant pathway controlling membrane trafficking of KCC4. Throughout the process, KCC4 is incorporated into membrane microdomains of lipid rafts and through the myosin Va-actin trafficking routes. (B) KCC4 functions as a plasma membrane scaffold protein with actin-binding protein, ezrin, in the assembly of cytoskeletal reorganization complex. (C) The possible mechanisms for the potent effect of KCC4 overexpression on cancer invasion and metastasis involve the modulation of MMP-2 activity, cell volume control, and the function as a plasma membrane scaffold with ezrin in the assembly of cytoskeletal reorganization complex. Among them, the regulated activation of MMP-2 and the functional role as a membrane scaffold seem to be associated with the ion transport activity. *Modified from Cancer Research 2009; 69:8585-8593 [24].*

bodies indicated that KCC3 is abundant in cervical carcinoma whereas KCC4 in metastatic cervical and ovarian cancer tissues [24, 49]. In the normal or noncancerous cervix, KCC3 protein expression is weak in both normal squamous epithelial and noncancerous stromal tissues [49]. In striking contrast, KCC3 protein is abundant in cervical carcinoma and in the tumor nest invaded deeply into stromal tissues. The expression level of KCC3 mRNA in tumor tissues is closely correlated with tumor size. Like KCC3, KCC4 protein is nearly undetectable in noncancerous cervical squamous epithelial tissues [24]. In contrast, primary cancerous tissues of cervix clearly express KCC4 protein at different levels. Additionally, KCC4 and its stimulators, EGF and IGF-1, are colocalized in the metastatic cancer tissues, suggesting the cooperation between KCC4 and EGF or IGF-1 in tumor metastasis. Higher KCC4 expression in tumor tissues is correlated with higher risk of tumor metastasis and cancer recurrence. Compared with low-grade KCC4 expression, primary tumors with high-grade KCC4 expression present the significantly higher percentage of parametrium invasion and pelvic lymph node metastasis, which are two major poor prognostic factors for early-stage cervical cancer. Consistently, increased KCC4 expression is associated with the poor clinical outcome, including increased percentage of cancer recurrence. Taken together, the findings from molecular qualitative and quantitative studies of KCC in surgical specimens suggest that the expression of KCC3, KCC4, and

their stimulators, EGF or IGF-1, exhibit a close association with clinical outcome of cancer patients. Therefore, KCC3, KCC4, EGF, and IGF-1 may be a panel of promising diagnostic biomarkers to predict cancer patient outcome.

Acknowledgments

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Abbreviations: EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; IGF-1, insulin-like growth factor-1; KCC, potassium chloride cotransporter; MMP, matrix metalloproteinase; RBC, red blood cell.

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