The up-regulation of KCC1 gene expression in cervical cancer cells by IGF-II through the ERK1/2MAPK and PI3K/AKT pathways and its significance

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Summary

Objective: To research the alternation effect of insulin-like growth factors-2 (IGF-II) on the expression of KCl co-transport-1 (KCC1) in the SiHa cells of cervical cancer, and to explore the activation of ERK1/2MAPK and PI3K/AKT signal transduction pathways during the expression process. *Method:* To apply semi-quantitative RT-PCR and Western blot analysis to measure changes in mRNA and protein expression of KCC1 after exposure to different concentrations of IGF-II for different time durations in the SiHa cells of cervical cancer. The change in protein expression of the ERK1/2 and AKT pathways is also measured. Furthermore, the protein expression variation in ERK1/2, AKT, and KCC1 is observed after the addition of a specific pathway blocker for the ERK1/2MAPK and PI3K/AKT pathways. *Results:* The mRNA and protein expression of KCC1 increases dramatically after the application of IGF-II on the SiHa cells, and shows a definite dosage-time dependence relationship. The protein phosphorylation is enhanced in the ERK1/2 and AKT pathways, where the protein activity increases. By adding a specific pathway blocker, the protein activity and phosphorylation of the two pathways are no longer promoted even under the effect of IGF-II. *Conclusion:* IGF-II can enhance KCC1 gene expression in cervical cancer cells through the ERK1/2MAPK and PI3K/AKT signal transduction pathways.

Key words: Insulin-like growth factor II; KCl Co-transport-1; Cervical cancer; RT-PCR, Western blot.

Introduction

Specific growth factors can significantly boost the invasion and metastatic ability of cancer cells, and this has become a serious problem to face in the treatment of malignant tumors, especially the important role of the insulin-like growth factor (IGF) family plays in the regulation of cell growth and differentiation, as well as inhibition of its apoptosis, which has recently become a focus point for researchers. IGF-II belongs to the family mentioned above, and can promote cancer activities by activating IGF-I receptors. KCl co-transport (KCC) is a cell surface membrane protein, divided into four sub-types, where KCC1, KCC3, and KCC4 can also induce K^+ and Cl^- ions' movement in pairs at the level of the cell membrane. Their activation plays an important role in cell volume regulation, epithelium transportation, and ionic balance. This study employs PCR and Western blot techniques to measure the mRNA and protein expression of KCC1 on the cell surface after the effect of IGF-II acting on the SiHa cells of cervical cancer, while the activation of signal transduction pathways is also explored.

Materials and Method

Research materials and source

SiHa cell strains of human cervical cancer (purchased from Shanghai Institute of Cell Biology) of low differentiated squamous epithelium tumor cells from females aged ≥ 55 years with pathological grade 2 were used [1].

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Research materials and equipment used: MEM culture media (US Gibco Company); fetal bovine serum (German Chrome Company); RNAiso test article (TaKaRa) and reverse transcription amplification test kit (TaKaRa RNA PCR Kit [AMV] Ver. 3.0) DRR019A (Dalianbao Bioengineering Co., LTD.); KCC1mRNA primer (synthesized by Dalianbao Bioengineering Co., LTD.); RT-PCR reaction equipment (GnenAmp PCR System 2400); the Image PC alpha 9 image analysis system; KCC1 goat polyclonal antibodies (KCC1(P-20): sc-25037; Santa Cruz Biology Co., LTD.); four antibodies, phospho-AKT (Ser 473), AKT, phospho-p44/42 MAP Kinase (Thr202/Tyr204), p44/42 MAP Kinase, and three blockers, PD98059, LY294002, and wortmannin (US Cell Signaling Company).

Cell culture

The SiHa cell strains of human cervical cancer were cultured by the standard set by the American Type Culture Collection, where MEM media (with non-essential amino acids and glutamine) was mixed with 10% of fetal bovine serum and breed for the next generation every two to three days.

Application of IGF-II on cells was done after 24 hours of non-serum culture in the following two approaches: same concentration at different times: for 2 ml of non-serum cell culture, IGF-II was added until the final concentration reached 50 ng/ml and then the effect was observed at 6 hr, 3 hr, 1 hr, 30 min, and 10 min time intervals. Different concentrations at the same time: for 2 ml of non-serum cell culture, different final concentrations (100, 50, and 10 ng/ml) of IGF-II were added and acted upon for a fixed time of six hours.

The co-application of IGF-II and specific pathway blocker was done 24 hours after culturing in the non-serum media; three bottles of equal quantities of cells were added with different concentrations (50 μM, 50 μM, and 1 μM) of PD98059

(ERK1/2MAPK pathway blocker), LY294002 (PI3K/AKT pathway blocker), and wortmannin (PI3K/AKT pathway blocker), respectively, and acting upon for one hour. After reaching the final concentration of 100 ng/ml, IGF-II was added. Collection of cells was done after one and six hours, respectively.

Reverse-transcription PCR (RT-PCR) was used to measure the mRNA expression of KCC1: TrizolTM test article was used to further extract the total cell RNA, and only 2 μg of the total was used for reverse transcription to synthesize the first cDNA. Equal quantities of cDNA were taken as a template for PCR to amplify the KCC1 gene, as well as housekeeping β-actin as an internal reference. The KCC1 primers were designed by Primer Designer 5.0 software, and the sequence was as the follows:
- KCC1, 278 bp, 58°C
- Upstream: 5'-TGGGACCATTTTCCTGACC-3'
- Downstream: 5'-CATGCTTCTCCACGATGTCAC-3'
- β-actine, 498 bp, 58°C
- Upstream: 5'-GTG GGG CGC CCC AGG CAC CA-3'
- Downstream: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'

The amplified product underwent electrophoresis on 1.5% agarose gel, with DNA Marker DL2000 as a standard reference for the positive agarose gel electrophoresis band. The gel imaging analysis system was used to scan and measure the absorption value of each band (A), and the relative intensity (basically the intensity) of KCC1 expression is represented by the ratio of KCC1 and β-actin absorptions. The change in intensity in the mRNA expression of KCC1 was also analyzed after the effect of IGF-II.

The five protein expressions under the effect of IGF-II (KCC1, Erk1/2, phosphorylated ERK1/2, AKT, and phosphorylated AKT) and the KCC1 protein expression under the co-effect of IGF-II and blockers were measured by Western blot: The proteins were acquired by cell lysis, and isolated by electrophoresis under 15% SDS-polyacrylamide gel. For each well, 50 μg of sample was loaded, and then the routine procedures were followed which involved transferring, sealing, and incubating with corresponding primary and double-antibodies in a shaker under room temperature for two hours after electrophoresis. Then, the enhanced chemical luminescence colorization system was used for exposure, display, and fixation of images.

Image analysis: The Image PC alpha 9, image analysis system, was applied for the blotting results of RT-PCR and Western-blot, where the accumulative absorption rate was calculated for each band.

Statistical analysis method: The experimental results were analyzed by the SPSS ver. 13.0 software, where data are displayed in x ± s presentation, and $p < 0.05$ means statistical significance.

Results

IGF-II effect on KCC1 mRNA expression in cervical cancer cells

The RT-PCR semi-quantitative measurement was used to illustrate the KCC1 mRNA expression in SiHa cells of cervical cancer affected by IGF-II. As the concentration of IGF-II increases and its application time elongates, the mRNA concentration of KCC1 increases as well. In comparison with the blank control, concentrations of 100 mmol/l and 50 mmol/l of IGF-II had significantly higher KCC1 mRNA concentration ($p < 0.01$), as well as in the group with 10 mmol/l of IGF-II ($p < 0.01$). Also, the

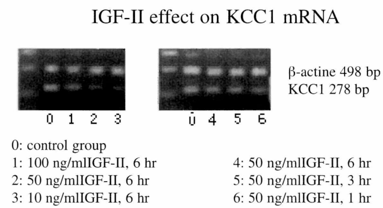

Figure 1. — RT-PCR.

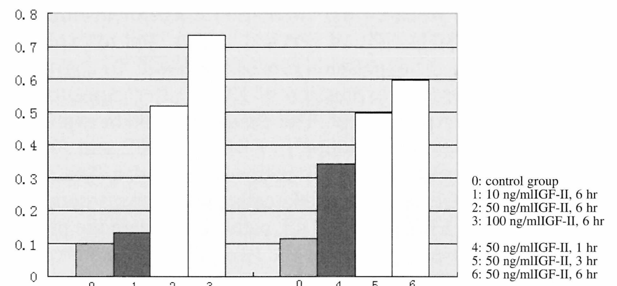

Figure 2. — IGF-II effect on KCC1 mRNA.

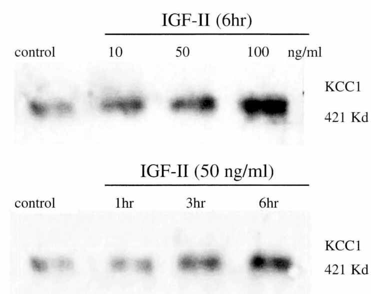

Figure 3. — 2 WB - IGF-II effect on KCCI protein expression.

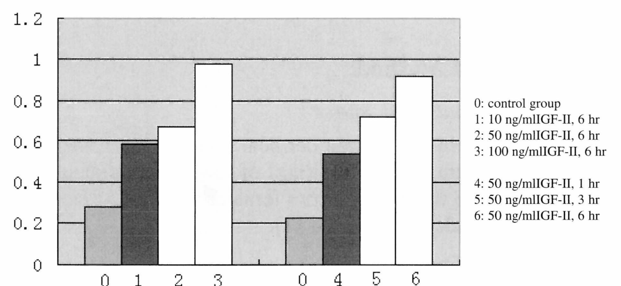

Figure 4. — IGF-II effect on KCC1 protein expression.

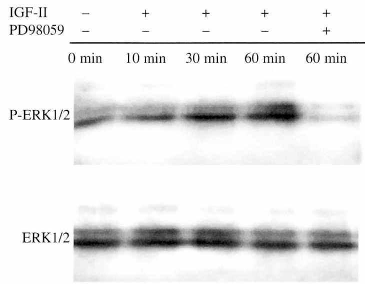

Figure 5. — IGF-II effect on P-ERK1/2 protein expression.

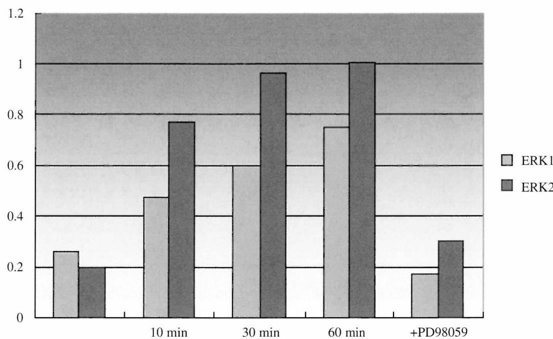

Figure 6. — IGF-II effect on P-ERK1/2 protein expression.

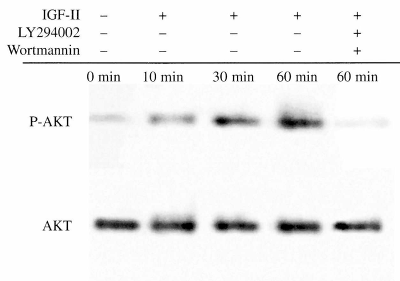

Figure 7. — IGF-II effect on P-AKT protein expression.

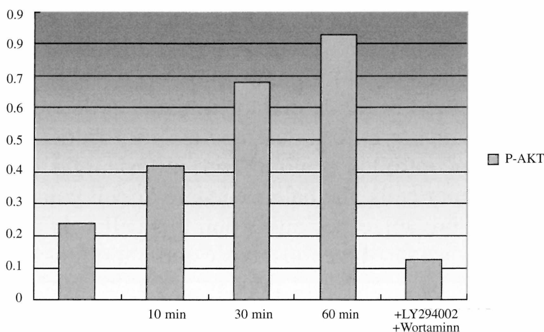

Figure 8. — IGF-II effect on P-AKT protein expression.

KCC1 mRNA expression increases immediately as soon as one hour after application of IGF-II, and continues to climb in an inclination trend as time elongates in three and six hours ($p < 0.01$). The result suggests that IGF-II indeed has an apparent influence on KCC1 mRNA expression in SiHa cells of cervical cancer, and this effect is dosage-time dependent.

IGF-II effect on KCC1 protein expression in cervical cancer cells

The Western blot was used to illustrate that the KCC1 protein expression in SiHa cells of cervical cancer affected by IGF-II has a similar pattern as the result of KCC1 mRNA expression, where the expression increased as the concentration and application time increased. In comparison to the blank control, the 100 ng/ml, 50 ng/ml, and 10 ng/ml of IGF-II groups had the KCC1 concentration increased ($p < 0.05$). Also, KCC1 protein expression increased immediately, as soon as one hour after the application of IGF-II, and continued to climb in an inclination trend as time elongated in three and six hours ($p < 0.05$). The result suggests that IGF-II indeed has an apparent influence on KCC1 protein expression in SiHa cells of cervical cancer, and this effect is dosage-time dependent.

IGF-II effect on the activation of ERK1/2MAPK and PI3K/AKT signal transduction pathways in cervical cancer cells

The Western blot was used to illustrate that phosphorylation of ERK1/2 and AKT proteins are enhanced in SiHa cells of cervical cancer affected by IGF-II. As the IGF-II concentration increased and application time elongated, the phosphorylation of two proteins increased, showing a similar trend as KCC1 protein expression. After addition of specific pathway blockers for these two pathways and with a maximum concentration and maximum application time, an increase in KCC1 protein expression was not observed by Western blot measurement.

Discussion

Cervical cancer is the second most frequently occurring tumor in the female reproductive system worldwide, and its incidence rate is rapidly increasing each year. The onset age is also getting younger as well, and now, it has become a major threat to female health. In many basic researches concerning cervical cancer, the infection of human papillomavirus (HPV) as a cause has been identified and proved [2]. However, in females infected with HPV, only a few cases have developed into cervical cancer, which suggests that there are many other factors involved other than HPV infection.

Currently, the pathogenesis of cervical cancer is one of the hot research topics and clinical researchers are primarily focusing on the specific growth factor dependent proliferation and metastatic ability, of which IGF is one of them. It is a growth hormone (GH), an endocrine secreted factor, that induces metabolism and synthesis, but is also

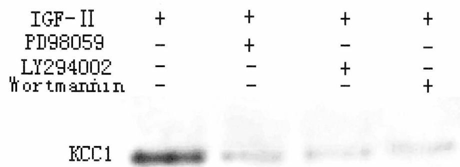

Figure 9. — The effect of three specific pathway blockers on KCC1 protein expression.

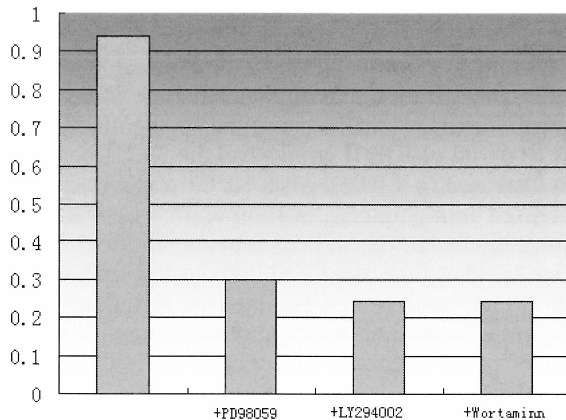

Figure 10. — Effect of three specific pathway blockers on KCC1 protein expression.

Table 1. — *1RT-PCR*
IGF-II effect on the change in KCC1 mRNA expression.

Grouping (IGF-II concentration, application time)	Relative A-value ($\bar{X} \pm s$)
Control group	0.11 ± 0.04
10 ng/ml; 6 hr	0.13 ± 0.17*
50 ng/ml; 6 hr	0.52 ± 0.11*
100 ng/ml; 6 hr	0.73 ± 0.16*
50 ng/ml; 1 hr	0.34 ± 0.12*
50 ng/ml; 3 hr	0.59 ± 0.17*
50 ng/ml; 6 hr	0.69 ± 0.23*

# p < 0.05, compared with control group; * p < 0.01, compared with control group.

Table 2. — *2 WB*
IGF-II effect on the change in KCC1 mRNA expression.

Grouping (IGF-II concentration, application time)	Relative A-value ($\bar{X} \pm s$)
Control group	0.28 ± 0.002
10 ng/ml; 6 hr	0.58 ± 0.07*
50 ng/ml; 6 hr	0.67 ± 0.03*
100 ng/ml; 6 hr	0.99 ± 0.11*
50 ng/ml; 1 hr	0.52 ± 0.07*
50 ng/ml; 3 hr	0.75 ± 0.11*
50 ng/ml; 6 hr	0.94 ± 0.34*

* p < 0.01, compared with control group.

a polypeptide that controls cell growth, differentiation, and apoptosis by paracrine and autocrine approaches [3]. IGF-II also belongs to the IGF family, and is more closely related to cervical cancer development than IGF-I, and thus has attracted more attention. It can activate the insulin-like growth factor receptor I (IGF-IR) by paracrine and autocrine approaches and exploit its ability for proliferation and metastasis of malignant tumors [4-6]. IGF receptor belongs to the family of tyrosine kinase growth factor receptor [3]. After stimulation and the tyrosine kinase is activated, an extra cellular signal is transducted inward the cell, to promote mitosis, protein synthesis, cell locomotion, and inhibition of apoptosis. In the downstream of the signal transduction pathway of tyrosine kinase activation, ERK1/2MAPK and PI3K-AKT/PKB pathways are the most understood intranuclear signal transductions for stimulating and enhancing transcription, where ERK (P42/44MAPK plays an important regulatory role in the differentiation and proliferation of tumor cells [7].

Recent researches have shown that KCl co-transport (KCC) is one of necessary factors that causes in-situ invasion, a cellular chemotaxis across the basal lamina, as a result of stimulation by insulin [8]. The activation of the growth factor-mediated ERK pathway requires participation of potassium ions in human medulloblastoma ML-1, which data have shown the efflux of K^+ ions is essential for the ERK1/2MAPK, as well as having an important role in the MAPK pathway. IGF-I and IGF-II can both activate IGF-I receptor to start the efflux of K^+ mediated by KCC, and thus KCC will be the downstream factor for IGFs to enhance the proliferation and metastasis ability of malignant tumors [9].

KCC, also known as volume-sensitive KCC, has four sub-types, which except for KCC2's neural specificity that inhibits the maturation of inhibitory NT, GABA, by controlling concentration of Cl^- [10], the others, KCC1, KCC3, and KCC4 have osmotic pressure sensitivity. The primary function is to regulate cell volume under the condition of changed homeostasis where osmotic pressure is unbalanced to cause inflation of cell volume [11, 12]. Many researchers have illustrated that KCC remains inactive when cervical epithelium is under normal physiological status. When cells undergo low-osmotic shock, KCC is slightly increased, while KCC expression skyrockets when cells are under high-osmotic shock [13].

The proliferation and invasion of cancer cells are complex processes involved with mitosis, cell locomotion and localization, as well as participation of other secreting enzymes. Cellular growth and locomotion are in fact a modulation of cell volume, and are regulated by specific mechanisms. Malignant tumor cells usually have higher a metabolic rate than normal cells, and also possess a higher mitosis rate and ability for locomotion. During uncontrolled proliferation, mitosis, and locomotion of tumor cells, the balanced state of cell volume regulation is broken, especially when this cell volume regulation is considered the most basic and important function for mammals, which can prevent possible swelling and shock due to osmotic pressure changes. Most cells have a complete cell volume regulating mechanism to counteract, and this is called regulatory volume decrease (RVD)

[14]. The induction of RVD relies primarily on the opening of K^+ and Cl^- ion channels [15,16]. Another important pathway is the co-transport of K^+ and Cl^- (KCC) that exists on the RBC and epithelium, where KCC can simultaneously transport both ions out of the cell membrane while working with other ion transport channels to regulate tumor cell volume, as proven by the radioactive $^{86}Rb^+$ labeled K^+ efflux experiment [17].

Research shows that KCC polypeptide remains at low basal expression in normal cervical tissue, while it has higher expression in tumor tissues. Also, some researchers have shown that there is co-localization of IGF-I and KCC expressions in cervical cancer and ovarian cancer by the immunofluorescent analysis of surgical specimens, which suggests that IGF by paracrine and autocrine approaches can stimulate KCC expression [18]. These all explain that KCC high expression is probably a selective advantage for abnormal proliferation and invasion of cervical tumor cells, and these processes are also closely related to cell volume regulation. Moreover, IGF can be the upstream regulatory factor that can finally activate KCC expression and thus the promotion effect on malignant tumor cell growth through various signal transduction pathways.

In this study, we applied different concentrations of IGF-II for different time durations on the SiHa cells of human cervical cancer, and observed the change in KCC expression, as well as the channel protein activity. Results show, in comparison to blank control and under the effect of IGF-II, KCC1 mRNA and protein expressions increased, while also illustrating a dosage-time dependency. Furthermore, phosphorylated ERK1/2 and AKT proteins also had in an inclination trend, explaining how IGF-II up-regulates the KCC mRNA expression by ERK1/2MAPK and PI3K/AKT pathways. As a result, KCC is possibly the downstream regulatory factor for the promotion of cervical cancer by IGF-II.

Many studies have shown that the invasion of cervical cancer cells can be stopped to some degree if the expression of KCC can be inhibited or inactivated. In 2000, Shen *et al.* used DIOA (a KCC blocker) to inhibit KCC; the RVD process was stopped, whereas volume-sensitive Rb efflux had decreased, and this phenomenon was dosage-dependent [17]. Function analysis of activated KCC suggests that the removal of 117 amino acids at the N-terminal end of KCC1 is enough to produce a negative impact to establish a human cervical cancer cell strain with function loss of missing KCC genotype, which as a result, the proliferation and invasion abilities dramatically decrease [19]. In 2004, Shen *et al.* employed specific pathway blockers for ERK1/2MAPK and PI3K/AKT and discovered that while there was cellular channel protein expression, the supposedly increasing KCC protein expression under the influence of IGF-I, was not observed. Also, application of the siRNA interference method to block the ERK1/2MAPK signal transduction pathway produced similar results as above [18]. This suggests that through blockage of certain transduction pathways, the KCC protein expression under IGFs effect will not increase.

In this study, we used one ERK1/2MAPK and two PI3K/AKT specific pathway blockers on cervical cancer cells for one hour. Afterwards, IGF-II was given. In comparison with the reference group, KCC1 protein expression did not increase. This is similar to other results published by Shen *et al.* [18]. It proves that specific inhibition on signal transduction can to some degree stop the up-regulation effect of IGF-II on KCC1 expression in cervical cancer cells. This has also brought about a new insight, where the minimization of KCC activity or even inhibition of its expression may become a new treatment strategy for IGF-dependent proliferation and invasion of cervical cancer.

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