



Overexpression of xCT induces up-regulation of 14-3-3 β in Kaposi's sarcoma

Yan ZENG* \dagger , Yan LI \dagger , Ri-Sheng CHEN \dagger , Xin HE \dagger , Lei YANG \ddagger ¹ and Wei LI \dagger ¹

*Key Laboratory of Xinjiang Endemic and Ethnic Disease, Department of Biochemistry, School of Medicine, Shihezi University, Xinjiang 832000, People's Republic of China, \dagger Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China, and \ddagger College of Public Health, Hangzhou Normal University, Zhejiang 310036, People's Republic of China

Synopsis

KSHV (Kaposi's sarcoma-associated herpesvirus), or HHV-8 (human herpesvirus 8), is associated with the pathogenesis of KS, the most common AIDS-related malignancy. xCT (functional subunit of the cystine/glutamate transporter x_c^- system) is known as the HHV-8 fusion-entry receptor as well as an oncogenic protein. How the xCT triggers the signal transduction of HHV-8 infection and the cell proliferation remains incomplete. We found that xCT was overexpressed in KS tissues and HHV-8-positive BCBL-1 cells. When xCT cDNA plasmids were transfected into the HHV-8-negative BJAB cells, the expression of 14-3-3 β and cell growth rate were increased. In contrast, the expression of 14-3-3 β and the cell growth rate of HHV-8-positive BCBL-1 cells were suppressed by either xCT siRNA (short interfering RNA) or an xCT inhibitor, sulfasalazine. These results suggest that 14-3-3 β is a downstream effector of xCT in KS to mediate the cell proliferation.

Key words: human herpesvirus 8 (HHV-8), Kaposi's sarcoma, 14-3-3 β , xCT

INTRODUCTION

KS (Kaposi's sarcoma) was first described by a Hungarian dermatologist, Moritz Kaposi, in 1872. It grows slowly and exhibits lesions on skin or internal organs. Skin lesions are the major symptoms of most KS patients [1]. KS is categorized into four types: CKS (classic KS), AIDS-KS (AIDS-associated KS), endemic KS and immunosuppression-associated or transplantation-associated KS [2]. The frequency of KS differs in regions and in populations. It occurs mostly in Jews and people of Mediterranean origin [3]. In China, Xinjiang is the region with the highest incidence of KS [4]. CKS has been reported mainly in Uygur and Kazakh populations in Xinjiang. In 1994, Chang et al. [5] identified DNA fragments of a previously unrecognized herpesvirus in a KS patient with AIDS, which was called KSHV (Kaposi's sarcoma-associated herpesvirus; also known as HHV-8 (human herpesvirus 8)). KSHV has now been detected in all kinds of KS. HHV-8 is regarded as an important causative agent of KS. Currently, the knowledge about the pathogenesis of KS is quite limited due to the complexity of signalling pathways for HHV-8 infection and KS formation.

The 14-3-3 family is a highly conserved, ubiquitously expressed protein family [6–8]. In mammals, there are at least seven isoforms: β , γ , ϵ , η , σ , ζ and τ . 14-3-3 proteins are involved in multiple processes including cell growth, survival and differentiation. 14-3-3 regulates cell growth through its interaction with Raf-1. In the MAPK (mitogen-activated protein kinase) signalling cascade, Raf-1 [MAP3K (MAPK kinase kinase)] activates MEK1 [MAPK/ERK (extracellular-signal-regulated kinase) 1] and MEK2 [also known as MAPKK (MAPK kinase), or ERK activator kinase]. ERK is activated by MEK1 and MEK2. ERK in turn activates downstream MAPK to regulate gene expression [9]. In our previous studies, we found that 14-3-3 β [encoded by *YWHA*B (tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, beta polypeptide)] is highly expressed in the KS tumour tissues compared with adjacent normal tissues from KS patients [10]. This suggests that 14-3-3 β may play a role in the tumorigenesis of KS. However, the 14-3-3 β upstream signalling pathway remains incomplete.

System x_c^- , consisting of a catalytic light chain xCT (functional subunit of the cystine/glutamate transporter x_c^- system) [or SLC7A11 (solute carrier family 7 member 11)] and a regulatory heavy chain (4F2hc), transports cystine into cells in exchange for

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KS, Kaposi's sarcoma; AIDS-KS, AIDS-associated KS; CKS, classic KS; FBS, fetal bovine serum; HHV-8, human herpesvirus 8; KSHV, Kaposi's sarcoma-associated herpesvirus; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MEK1, MAPK/ERK (extracellular-signal-regulated kinase) 1; RT-PCR, reverse transcription-PCR; SASP, sulfasalazine; siRNA, short interfering RNA; SLC7A11, solute carrier family 7 member 11; *YWHA*B, tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, beta polypeptide; xCT, functional subunit of the cystine/glutamate transporter x_c^- system.

¹Correspondence may be addressed to either of these authors (email wli@genetics.ac.cn or yanglei62@yahoo.com).

the release of glutamate at a 1:1 ratio. Within the cell, cystine is rapidly reduced to cysteine, the rate-limiting substrate for glutathione synthesis. System x_c^- plays an important role in antioxidant by regulating the intracellular glutathione level [11,12]. Cystine uptake in human cancer cells is mainly dependent on system x_c^- . It has been shown that xCT is highly expressed in multiple cancers such as hepatoma, lymphoma, glioma and lung, colon, breast, prostate and pancreatic cancer cells, which renders xCT a potential drug target for cancer therapy [13]. Previously, it has been reported that xCT is the HHV-8 fusion-entry receptor [14]. HHV-8 induces ERK1/2 to modulate the initiation of viral gene expression and host cell gene expression [15]. Thus it is plausible to postulate that HHV-8 entry hijacks the ERK-mediated MAPK signalling pathway to promote cell proliferation in KS. Thus xCT may play a dual role in HHV-8-induced KS formation, as the HHV-8 receptor and as an oncoprotein. We report here that xCT induces 14-3-3 β overexpression in KS, for the first time establishing a link between xCT and the ERK-mediated MAPK signalling pathway.

MATERIALS AND METHODS

Tissue samples

Seventeen KS specimens, obtained from 1996 to 2008, were collected by the Key Laboratory of Xinjiang Endemic and Ethnic Diseases in Shihezi University, China. Ten patients with CKS, four patients with AIDS-KS and three patients with renal transplantation-associated KS were included in the present study. All patients were HHV-8 positive. Skin samples of 17 healthy subjects who had plastic surgery were used as a control group. The present study was approved by the Ethics Committee of Shihezi University, and all the participants provided written informed consent. The tumour tissues and tumour-adjacent normal tissues from KS patients were freshly prepared. Each sample was cut into two pieces; one was stored in liquid nitrogen for extracting total RNA and was used for the real-time fluorescent quantitative PCR tests and the other was fixed in formalin and was embedded in paraffin for immunohistochemistry studies.

Cell lines and cell culture

The melanocyte line from $xCT^{-/-}$ mice (*sut*) and the melanocyte line from wild-type mice ($xCT^{+/+}$) were established in Dr Dorothy Bennett's laboratory (Division of Basic Medical Science, St George's, University of London, London, U.K.) and cultured as described in [16]. The medium for both $xCT^{+/+}$ and $xCT^{-/-}$ melanocytes was supplemented with 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, U.S.A.) [17]. Human BCBL-1 and BJAB cell lines were kindly provided by Wuhan Institute of Virology, Chinese Academy of Sciences. These cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS (fetal bovine serum; Sigma-Aldrich). HeLa cells were grown in Dulbecco's modified Eagle's medium plus 10% FBS. All cell lines were cultured at 37°C with 5% CO₂.

Real-time fluorescent quantitative PCR

Total RNA was isolated from skin tissues after the removal of genomic DNA by DNase I treatment and subjected to reverse transcription. Real-time fluorescent quantitative PCR was conducted on 100 ng of cDNA from the 17 KS tissues and 17 KS-adjacent normal tissues. The housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was selected for normalization [18]. An iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, U.S.A.) was used in conjunction with a QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. To calculate the relative expression for each gene, the $2^{-\Delta\Delta C_t}$ method was used as the average C_t (threshold cycle value) value for the housekeeping gene [19].

Immunohistochemistry

Detection of 5 μ m sections of 17 KS tumours was performed by the immunohistochemical Envision technique. The tissue array slide was deparaffinized and rehydrated before H₂O₂ treatment for 30 min at room temperature (25°C). Antigen retrieval was performed by microwave heating sections in 10 mmol/l sodium citrate buffer (pH 6.0) for 5 min. After blocking of non-specific binding, mouse anti-14-3-3 β monoclonal antibody (H8, SC-1657, Santa Cruz Biotechnology) was diluted 1:300 and rabbit anti-xCT polyclonal antibody was diluted 1:100, and incubated at 4°C overnight. Normal human skin tissue sections were used as controls. Immunostained sections of KS tissues and normal human skin tissues were scored based on the percentage of immuno-positive cells (0–100) and staining intensity (0–4).

RNA interference

The specific siRNA (short interfering RNA; Genechem, Shanghai, China) targeted to different regions of *SLC7A11* mRNA were synthesized and tested for efficiency in knockdown of xCT expression [17]. HeLa and BCBL-1 cells were transfected with siRNAs at 120 pmol each using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, U.S.A.), with the final concentration of siRNA being 100 nM, and collected for assay at 48 h post-transfection.

Western blotting

Cell lysates were prepared as described previously [17]. Protein samples in supernatant (20 μ g per lane) were subjected to SDS/PAGE and transferred on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). The PVDF membrane was incubated for 1 h in PBS containing 5% fat-free dried milk, and then incubated with mouse monoclonal antibody to 14-3-3 β (1:7000) or β -actin (1:5000), or rabbit polyclonal antibody to xCT (1:2000) or ERK1/2 (1:10000) at 4°C overnight, followed by a 2 h incubation with horseradish peroxidase-conjugated goat anti-mouse (1:5000) or goat anti-rabbit IgG (1:5000). We prepared the xCT antiserum immunized with GST (glutathione transferase)-xCT antigen. All the other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Protein bands were

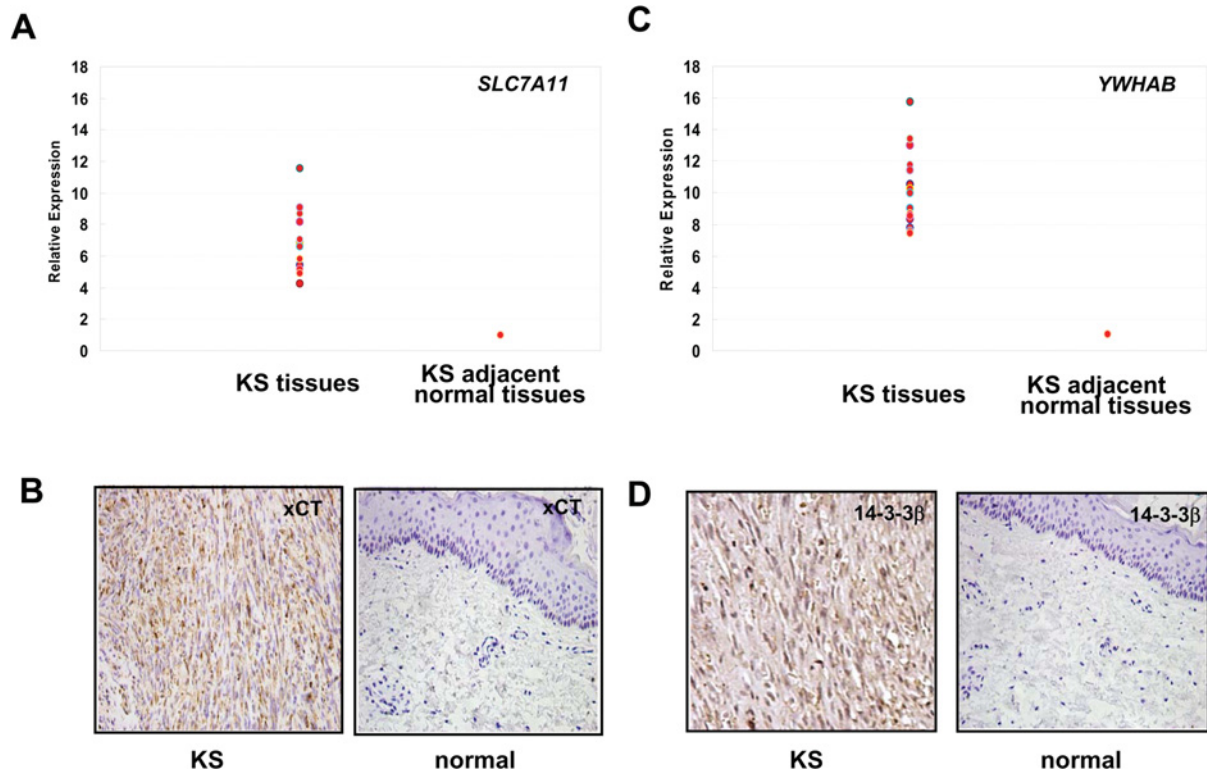


Figure 1 Higher expression of xCT in KS tissues

(A, C) Real-time fluorescent quantitative PCR detecting *SLC7A11* and *YWHAB* expression in KS and KS-adjacent normal tissues. *SLC7A11* and *YWHAB* mRNA levels in 17 different KS tissues were normalized to *GAPDH* and plotted as fold versus that in KS-adjacent normal tissues of the same patients. Results shown are the means for three independent experiments. (B, D) Images (magnification, $\times 400$) of immunohistochemical staining by xCT and 14-3-3 β in KS and normal skin tissues. xCT or 14-3-3 β immunoreactivities appear as a homogeneously brown staining in the cytoplasm. In contrast, the normal control shows negative or weak signals.

detected with ECL[®] Plus chemiluminescent reagent (GE Healthcare). The intensities of the bands obtained were determined by an Alpha Imager 2000 densitometer (Alpha Innotech, San Leandro, CA, U.S.A.). Each densitometric value was expressed as mean \pm S.D.

Survival rate assay

In the cell survival rate assay, BCBL-1 cells transfected with *SLC7A11* siRNA or control siRNA were seeded on to 96-well plates at a density of $2 \times 10^4/\text{cm}^2$ with RPMI 1640 medium supplemented with 10% FBS for 24 h. Cells were quantified by a CCK-8 kit (Dojindo, Kumamoto, Japan). A 10 μl portion of CCK-8 was added into each well and the attenuation (D) at 450 nm was measured. Wells with cell medium and CCK-8 solution served as a blank control, whereas wells seeded with BCBL-1 cells transfected with non-silencing siRNA served as a negative control. The cell survival rate was calculated as (test-blank control)/(negative control-blank control) $\times 100\%$. All experiments were performed in triplicate wells and repeated three times.

Statistics

Quantitative data in paired groups were analysed using a Student's t test. Qualitative data were compared using a χ^2 test.

RESULTS

xCT is highly expressed in KS tissues

As xCT may play a dual role (HHV-8 receptor and oncogene) in HHV-8-induced KS formation, we hypothesize that xCT may serve as an upstream candidate that is responsible for the 14-3-3 β up-regulation in KS. Both *SLC7A11* and *YWHAB* were overexpressed in KS tissues by real-time PCR (Figures 1A and 1C) and immunohistochemical staining (Figures 1B and 1D). Real-time PCR analyses showed that the *SLC7A11* expression in KS tissues was 6.2-fold (on average) higher than that in KS-adjacent normal tissues (Figure 1A). Likewise, the *YWHAB* expression in KS tissues was 9.5-fold (on average) higher than that in KS-adjacent normal tissues (Figure 1C). xCT has been found to be

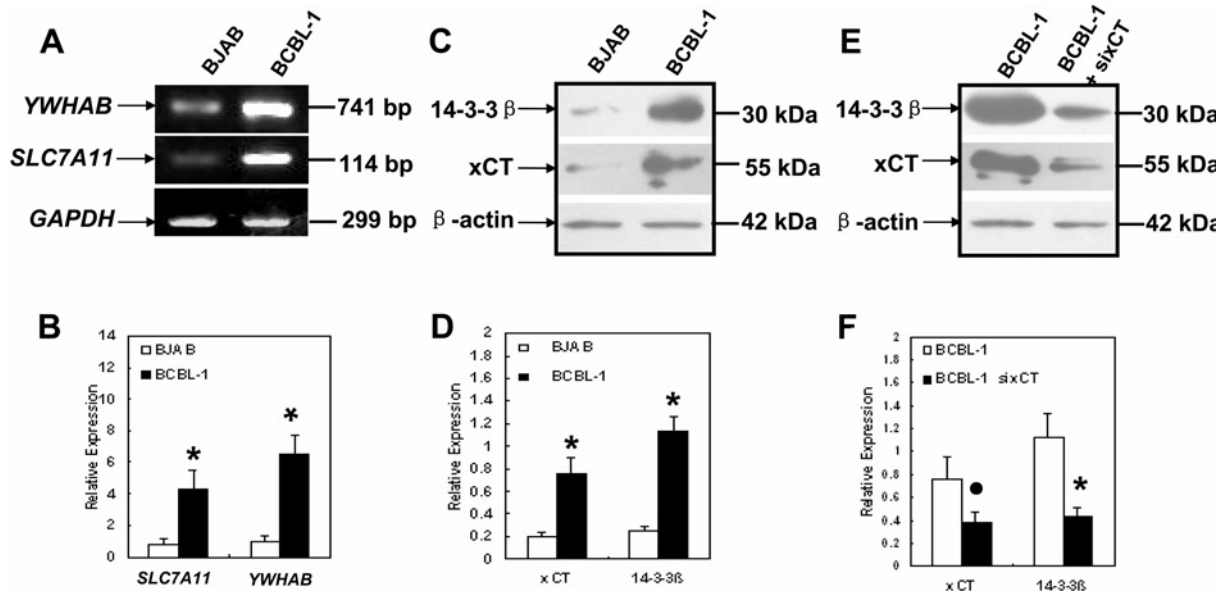


Figure 2 The expression of 14-3-3 β correlates with xCT in different treatments or cell lines

(A) BJAB (HHV-8⁻) and BCBL-1 (HHV-8⁺) cells were harvested and total RNA was extracted for semi-quantitative RT-PCR detection of the expression of *SLC7A11* and *YWHAB*. *GAPDH* was used as an internal control. (B) Real-time PCR detection of *SLC7A11* and *YWHAB* mRNA levels in BJAB and BCBL-1 cells. After normalization to *GAPDH*, *SLC7A11* and *YWHAB* mRNA expression in BCBL-1 cells was plotted as fold versus the BJAB control. Results shown are the means \pm S.D. for three independent experiments performed in triplicate (* $P < 0.01$). (C) BJAB and BCBL-1 cells were lysed and subjected to Western-blot analysis. β -Actin was used as a loading control. (D) Normalized percentages of the band intensities shown in (C) were calculated. Results shown are the means \pm S.D. for three independent experiments (* $P < 0.01$). (E) BCBL-1 and BCBL-1 cells transfected with *SLC7A11* siRNAs (sixCT) were lysed and subjected to Western-blot analysis. β -Actin was used as a loading control. (F) Normalized percentages of the band intensities shown in (E) were calculated. Results shown are the means \pm S.D. for three independent experiments (* $P < 0.01$, $\bullet P < 0.05$).

up-regulated in a variety of cancer cells [13], and these findings first revealed that xCT is overexpressed in KS tissues. To further establish the correlation between xCT and 14-3-3 β , we overexpressed or down-regulated the xCT activity to see whether 14-3-3 β changes accordingly in the following studies.

xCT induces the expression of 14-3-3 β

BCBL-1 (HHV-8⁺) cells promote lymphoma formation in SCID (severe combined immunodeficiency) mice, mimicking the features of KS cells [20]. We therefore determined whether 14-3-3 β and xCT are overexpressed in BCBL-1 cells. Indeed, the expression of 14-3-3 β and xCT in BCBL-1 cells was higher than that in BJAB (HHV-8⁻) cells (Figures 2A–2D). Real-time quantitative RT-PCR (reverse transcription-PCR) analyses showed that the mRNA expression of *SLC7A11* and *YWHAB* in BCBL-1 cells was 5.4- and 6.5-fold higher than that in BJAB cells respectively (Figure 2B). Western-blot analyses showed that the protein levels of xCT and 14-3-3 β in BCBL-1 cells were 3.8- and 4.6-fold higher than that in BJAB cells respectively (Figure 2D). Furthermore, we found that the expression of 14-3-3 β was suppressed when xCT was knocked down in BCBL-1 (HHV-8⁺) cells by the transfection of *SLC7A11* siRNA (Figures 2E and 2F). *SLC7A11* siRNA effectively down-regulated xCT and 14-3-3 β expression

in BCBL-1 cells, resulting in 52% reduction in xCT and 39% reduction in 14-3-3 β at protein levels (Figure 2F).

We next measured the effect of disruption of xCT on the cell growth of the BCBL-1 cells. The survival rate of BCBL-1 cells transfected with *SLC7A11* siRNA or treated with SASP (sulfasalazine) was significantly lower than that in the control cells. The survival rate of BCBL-1 cells transfected with *SLC7A11* siRNA was 45% lower than that in the cells transfected with non-silencing siRNA (Figure 3A). Similarly, the survival rate of BCBL-1 cells treated with 0.25 mM SASP was 53% lower than that in the untreated cells (Figure 3B). On the other hand, the survival rate of BJAB cells transfected with *SLC7A11* expression plasmid pCDNA3-HA-hxCT was significantly higher than that in the cells transfected with empty vector. The survival rate of BJAB cells transfected with xCT expression plasmid pCDNA3-HA-hxCT was 157% higher than that in the cells transfected with empty vector (Figure 3C). These results are the first to establish a correlation between xCT and 14-3-3 β in promoting cell growth.

xCT promotes cell growth through the 14-3-3 β -mediated ERK activation pathway

In the MAPK signalling pathway, 14-3-3 β regulates cell growth through its interaction with Raf-1, whereas Raf-1 activates MEK1

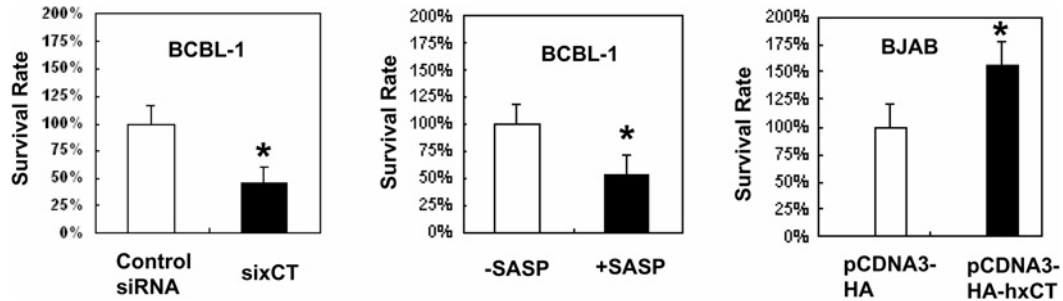


Figure 3 Disruption of xCT affects the KS cell growth rates

Survival rates are shown in BCBL-1 cells transfected with *SLC7A11* siRNA (sixCT, left), or treated with 0.25 mM SASP (middle), or in BJAB cells transfected with pCDNA3-HA-hxCT (right). After normalization to the control, the survival rates are presented as means \pm S.D. for three experiments performed in triplicates (* $P < 0.01$).

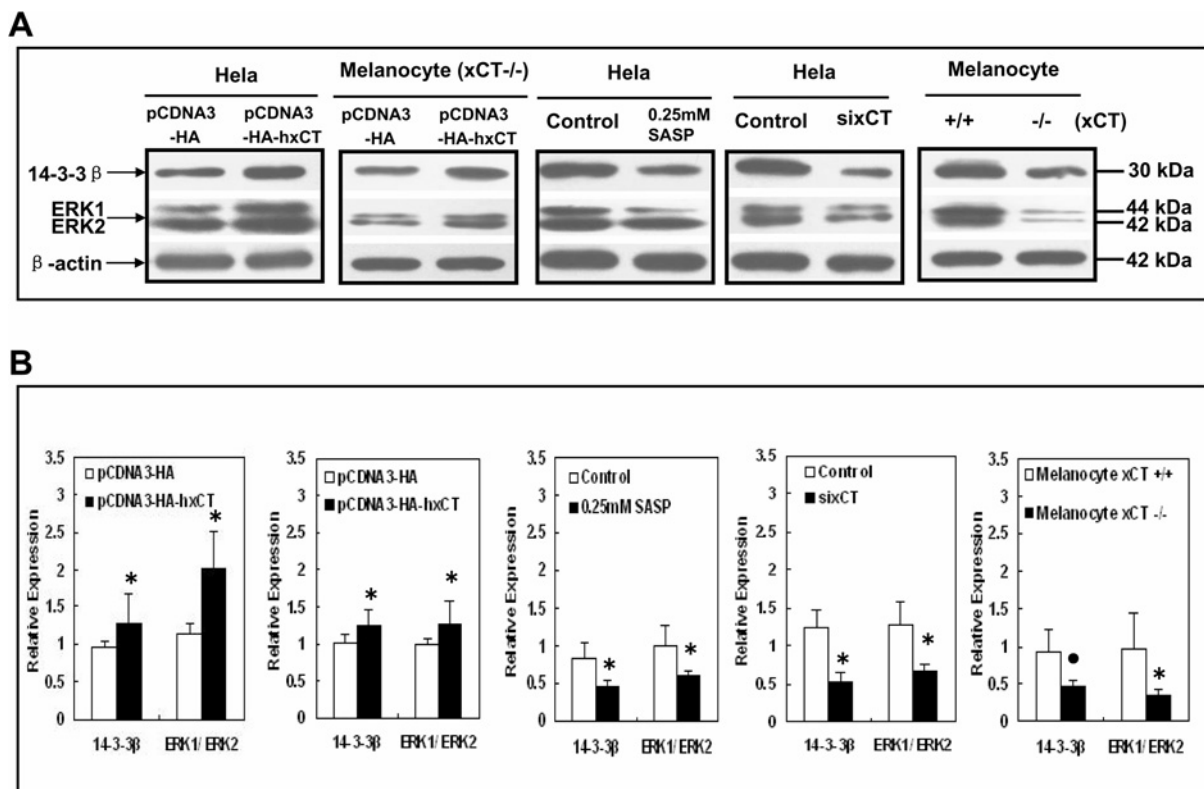


Figure 4 The expression of ERK1/2 correlates with 14-3-3β in different treatments

(A) xCT^{+/+}, xCT^{-/-} melanocyte or HeLa cells were treated with SASP, *SLC7A11* siRNA (sixCT) or pCDNA3-HA-hxCT. β-Actin was used as a loading control. (B) Normalized percentages, which are explained in the text, of the band intensities shown in (A) were calculated and the results shown are the means \pm S.D. for three independent experiments (* $P < 0.01$, ● $P < 0.05$).

and MEK2 (also known as MAPKK, or ERK activator kinase). ERK is in turn activated by MEK1 and MEK2 [9]. To determine whether the overexpression of xCT triggers the 14-3-3β-mediated ERK activation, we measured the expression levels of both 14-3-3β and ERK1/2 after the cells were transfected with pCDNA3-HA-hxCT plasmids (Figure 4). We found that the expression levels of both 14-3-3β and ERK1/2 were higher in the pCDNA3-HA-hxCT-treated HeLa cells or xCT^{-/-} melano-

cytes than those in the untreated cells. Western-blot analyses showed that the expression levels of 14-3-3β and ERK1/2 in HeLa cells transfected with pCDNA3-HA-hxCT plasmids were 134 and 178% higher than those in the cells transfected with empty vector respectively. Likewise, the expression levels of 14-3-3β and ERK1/2 in xCT^{-/-} melanocytes transfected with pCDNA3-HA-hxCT plasmids were 122 and 127% higher than those in the cells transfected with empty vector respectively.



Consistently, the expression levels of both 14-3-3 β and ERK1/2 were lower in the HeLa cells treated with either SASP or *SLC7A11* siRNA than that in the untreated cells. The protein levels of 14-3-3 β and ERK1/2 in HeLa cells treated with 0.25 mM SASP were 54 and 59% lower than those in the untreated cells respectively. *SLC7A11* siRNA effectively down-regulated 14-3-3 β and ERK1/2 expression in HeLa cells, resulting in reductions of 43% in 14-3-3 β and 53% in ERK1/2 at the protein level. Similarly, the expression levels of 14-3-3 β and ERK1/2 in xCT^{-/-} melanocytes were reduced to 50 and 36% respectively in the xCT^{-/-} melanocytes compared with the wild-type xCT^{+/+} melanocytes. Taken together, these results suggest that xCT promotes cell proliferation through the 14-3-3 β -mediated ERK activation pathway.

DISCUSSION

xCT has been reported to be highly expressed in a variety of cancer cells [13]. In the present study, we report for the first time that xCT is overexpressed in KS. As xCT plays a dual role in mediating HHV-8 entry and cell proliferation, it may be an upstream key player in KS formation. We found that xCT may transduce its signal to 14-3-3 β to mediate the MAPK signalling cascade in cell proliferation.

Our previous studies have shown that xCT is involved in multiple cellular processes including pheomelanin production and cell proliferation [16], oxidative stress and endoplasmic reticulum stress [21] and cancer cell metastasis [17]. All these functions are related to cystine uptake as well as intracellular redox modulation. Cancer cells consume more cystine and exhibit higher xCT activity. It is intriguing how this signal is transduced to the 14-3-3 β -mediated MAPK signalling pathway. xCT forms a complex with 4F2hc (CD98, SLC3A2) to be a functional system x_c⁻. It has also been shown that 4F2hc binds to β 1 integrin and is involved in β 1 integrin-mediated signal cascades [22,23]. Both xCT and 4F2hc are clustered in the lipid rafts, where xCT associates with α 3 β 1 integrin via 4F2hc and thus leads to the activation of FAK (focal adhesion kinase) and Src, which activates PI3K (phosphoinositide 3-kinase) and Rho-GTPases, facilitating ERK activation [24]. This may explain why overexpression of xCT correlates with the activation of ERK. However, to discover the exact molecular link between xCT and 14-3-3 β requires further investigation.

The involvement of xCT in cancer cell proliferation and metastasis renders it a promising therapeutic target in cancer therapy [13]. Pharmacological inhibition of xCT remarkably inhibited proliferation of multiple cancer cells *in vitro* and attenuated tumour growth *in vivo* [13,17]. Consistently, we have demonstrated here that SASP inhibits the expression of 14-3-3 β and ERK1/2 in HeLa cells and inhibits the cell growth in HHV-8-positive BCBL-1 cells, further supporting its potential as a drug target in cancer therapy, including KS. Considering the easy accessibility of xCT by SASP at the plasma membrane and its upstream role in 14-3-3 β and ERK1/2, xCT is regarded as a better therapeutic

target of the ERK-mediated MAPK signalling pathway in cancer intervention.

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