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Overexpression of KAI1 inhibits retinoblastoma metastasis *in vitro*

HUI YAN[1*], XUNDA JI[1*], JING LI[1], LEI ZHANG[2] and PEIQUAN ZHAO[1]

[1]Department of Ophthalmology, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200092; [2]Department of Ophthalmology, Tongji University Affiliated Yangpu Hospital, Shanghai 200090, P.R. China

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Abstract. The present study aimed to investigate the expression of cluster of differentiation 82 (KAI1), a gene involved in the suppression of tumor metastasis, in human retinoblastoma (RB) tissue and to study the effect of KAI1 expression on RB cell migration and invasion. KAI1 expression was examined in 26 patients with non-invasive and invasive retinoblastoma using reverse transcription-quantitative polymerase chain reaction and western blot analysis. A lentiviral vector containing KAI1 cDNA was used to transfect the two RB cell lines, HXO-Rb44-Gl and Y79. Following successful transfection, the migratory and invasive capacity of the two RB cell lines was evaluated using a Transwell® migration assay. KAI1 expression was observed to be downregulated in invasive RB compared to non-invasive RB. The migratory and invasive capacities of KAI1 transfected cell lines were significantly decreased compared to those of the control cells. KAI1 may be involved in retinoblastoma metastasis, and increased expression of KAI1 significantly inhibits the metastatic ability of RB cells *in vitro*.

Introduction

Retinoblastoma (RB) is the most frequent retinal tumor worldwide and the most common primary intraocular malignancy in childhood, accounting for ~4% of all pediatric malignancies (1). The majority of patients with retinoblastoma are not diagnosed until the disease has reached an advanced stage or metastasis has occurred (2). Tumor cells are able to extend through the optic nerve, sclera and choroid to access the extraocular space, leading to life-threatening systemic metastasis (3,4). Metastatic RB has a high mortality rate due to a limited range of chemo- and radiotherapeutic treatments (5). Although intensive multimodal therapies, including high-dose chemotherapy with autologous hematopoietic stem cell rescue, are reported to be effective in the treatment of metastatic RB, it remains a life threatening disease (6).

Cluster of differentiation 82 (KAI1) was first identified in prostate cancer cells by Dong *et al* (7) and has a significant role in the inhibition of tumor metastasis. As a member of the transmembrane 4 superfamily, KAI1 contains four hydrophobic transmembrane domains and a large extracellular N-glycosylated structure, which interacts with integrin, epidermal growth factor receptor and other tetraspanins (8-10). KAI1 interactions are associated with cell-cell and cell-extracellular matrix interactions, as well as cell signaling and motility. Therefore, KAI1 may affect the invasion and metastasis of tumor cells (11). Decreased KAI1 expression is correlated with the development of tumor metastasis and poor prognosis in a wide variety of human malignancies, including prostate (12), breast (13), pancreatic (14) and lung cancer (15). Decreased expression of KAI1 was reported in metastatic human RB samples (16). However, the biological effect of KAI1 on RB cells remains unclear.

In the present study, KAI1 expression was analyzed in 26 human retinoblastoma samples, representing non-invasive and invasive stages. In addition, the *in vitro* effect of KAI1 on RB cell migration and invasion was studied in two RB cell lines.

Materials and methods

Ethics statement. The present study conformed to the Declaration of Helsinki and was approved by the Ethical Committee of the Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China). Written consent was obtained from the patients participating in the study.

Tissue samples. Samples were obtained from RB patients at Xinhua Hospital (Shanghai, China) between May 2010 and February 2013, who received no treatment during the enucleation procedure. In total, 14 eyes presenting with no RB invasion of the optic nerve or choroid were selected, representing the non-invasive group, and 12 eyes presenting with invasion of the optic nerve or choroid were taken, representing the invasive group.

Detection of KAI1 protein using western blot analysis. Tumor samples were ground into powder in liquid nitrogen. Total

Correspondence to: Dr Peiquan Zhao, Department of Ophthalmology, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, P.R. China
E-mail: zhaopeiquan@126.com

*Contributed equally

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protein was extracted using an ice-cold SDS cell-lysis buffer (BiYunTian, P0013G, Shanghai, China), containing protease inhibitors, and lysate was centrifuged (15,800 x *g* for 30 min at 4˚C) to remove debris. After quantitative analysis, all the protein samples were mixed with the loading buffer and then boiled with the burning water for 5 min. The protein samples were separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Chalfont, UK). The membrane was blocked in TBS and Tween® 20 with 5% skimmed milk for 90 min, followed by incubation with monoclonal mouse anti-KAI1 antibody (1:500, sc-4486 Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Following three cycles of washing with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:1,000, Jackson, 115-035-206, USA) at room temperature for 1 h. β-actin (1:2,000, Proteintech, 6008-1-Ig, USA) was used as an endogenous control. The resulting signals were measured using an enhanced chemiluminescence system. Band intensity was normalized to the value of β-actin (Image J, NIH, USA). All experiments were performed in triplicate.

Western blot analysis for protein samples in RB cells. RB cells incubated in mediums were collected and transferred into EP tube with the number of $1x10^6$ in each tube. After centrifuging (4˚C, 800 rpm, 10 min), each EP tube were added with an ice-cold SDS cell-lysis buffer (BiYunTian, P0013G, Shanghai, China), containing protease inhibitors, and lysate and were centrifuged (15,800 x *g* for 30 min at 4˚C) to remove debris. The following steps and experimental conditions were in consistent with the western blot analysis for tumor samples.

Measuring KAI1 gene expression using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RB tissues were ground into powder in liquid nitrogen and dissolved using a Trizol® reagent kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The MyiQ and iQ5 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), SYBR-Green kit and SYBRR Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan) were used to perform RT-qPCR and subsequently measure KAI1 mRNA expression. The primer pairs for KAI1 and GAPDH were as follows: KAI1 forward, 5'-TGTCCTGCAAACCTCCTCCA-3' and reverse, 5'-CCATGAGCATAGTGACTGCCC-3'; and GAPDH forward, 5'-CCATGGCACCGTCAAGGCTGA-3' and reverse, 5'-GGGCCATCCACAGTCTTCTGG-3'. Reaction parameters for RT-qPCR were as follows: 94˚C for 5 min followed by 40 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec. Each reaction was performed in duplicate. The relative mRNA levels of KAI1 and GAPDH were calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Cell lines and culture conditions. HXO-Rb44-Gl is a green fluorescent protein (GFP) and luciferase expressing HXO-Rb44 human RB cell line (18). The HXO-Rb44 cell line was originally established by the Department of Ophthalmology, Hunan Medical University, Xiangya Hospital, Changsha, China (19). The Y79 human RB cell was purchased from the American Type Culture Collection (Manassas, VA, USA). Non-adherent cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (both Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2 humidified air.

Construction of recombinant lentivirus expressing KAI1. pCMV-KAI1 was provided by Dr Dong of the National Institute of Health (Bethesda, MD, USA). The 753 bp KAI1 and the 849 bp enhanced GFP (EGFP) cDNA fragments were amplified from pCMV-KAI1 and pEGFP-N1 respectively using RT-PCR with the following primers: KAI1 forward, ATTCGCCTAGGACTAGTGATCAGCCACCATGGG CTCAGCCTGTATCAA and reverse, CGGGGTACTTGG GGACCTTGCTGT; EGFP forward, CAAGGTCCCCAA GTACCCCGGGGGTGGCGGAGGGTCTAGAATGGTGAG CAAGGGCGAG and reverse, TCGGCGGCCGCTTATTTG TCGTCATCATCCTTATA. The two fragments were fused together to form a 1585 bp KAI1-EGFP DNA fragment using PCR, and inserted into the puromycin-resistant lentiviral vector pMB-Puro (Neuronbiotech Co., Ltd., Shanghai, China), replacing the firefly luciferase gene. The recombinant plasmid pMB-Puro-KAI1-EGFP and the two 2nd generation packaging plasmids, psPAX2 and pMD2.G (Addgene, Inc., Cambridge, MA, USA), were introduced into 293T cells (donated by American type culture collection), and the resulting virus (Lenti-MPKG) was harvested and titrated. Briefly, $5x10^4$/well 293T cells were plated onto 6 well plate 1 day before infection. Subsequently, these cells were 10-fold serial dilutied. 3 days after infection, these cells were digested by EDTA solution and centrifuged. The titer of virus was determined by real-time qPCR (20,21). A control virus containing EGFP alone (Lenti-MPG) was generated in parallel.

Generation of stable Rb-KAI1 cells. HXO-Rb44-Gl cells and Y79 cells were infected with Lenti-MPKG to generate stable HXO-Rb44-Gl-KAI1$^+$ and Y79-KAI1$^+$ cells, respectively. The stable Rb-KAI1 cells were selected using 4 μg/ml puromycin. HXO-Rb44-Gl and Y79 cells were infected with Lenti-MPG to generate the stable control cell lines, HXO-Rb44-Gl-KAI1$^-$ and Y79-KAI1$^-$. Stable transduced cells were maintained in RPMI-1640 medium containing 10% FBS and 2 μg/ml puromycin.

Immunofluorescence staining analysis. Cultured cells were collected by centrifugation (15,800 x *g* for 15 min at 4˚C), washed with cold PBS and fixed in 4% paraformaldehyde. Fixed cells were subsequently blocked with PBS containing 3% bovine serum albumin (BiYunTian, ST023, Shanghai, China) for 1 h, and incubated with monoclonal mouse anti-KAI1 antibody (1:100) overnight at 4˚C. Cells were washed with PBS and centrifuged (3,600 x *g* for 15 min at 4˚C) prior to incubation with rhodamine-tagged secondary antibody (BiYunTian, A-0568, Shanghai, China) at 37˚C for 1 h. Tagged cells were subsequently mounted with DAPI-containing mounting solution and observed using a fluorescence microscope.

Cell proliferation analysis. The Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to quantify cell growth. Cells (HXO-Rb44-GI-KAI1$^+$, HXO-Rb44-Gl-KAI1$^-$, HXO-Rb44-Gl; Y79-KAI1$^+$, Y79-KAI1$^-$, Y79) were seeded into 96-well plates at a density of $1x10^4$ cells per well in 100 μl

RPMI-1640 10% FBS. At the indicated time points (1 day, 2 day, 3 day, 4 day, 5 day, 6 day, 7 day), 10 μl CCK-8 was added to each well and the plate was incubated for 1 h at 37˚C. The optical absorption of each well at 450 nm was measured using a spectrophotometer.

Cell migration assay. The cell migration assay was performed using a Transwell® chamber and polyethylene terephthalate membrane (PET) insert (Corning Inc., Corning, NY, USA) with 8 μm pores. A total of 100 μl cells ($0.5x10^6$/ml) (HXO-Rb44-GI-KAI1$^+$, HXO-Rb44-Gl-KAI1$^-$, HXO-Rb44-Gl; Y79-KAI1$^+$, Y79-KAI1$^-$, Y79) and 200 μl serum free RPMI-1640 media were added to the top chamber. A total of 500 μl RPMI-1640 media with 10% FBS was added to the bottom chamber as a chemoattractant. Each cell line was plated in three duplicate wells. Following 24 h incubation (37˚C, 5% CO_2), the migratory cells in the bottom chambers were counted using a light microscope in five randomly selected visual fields.

Cell invasion assay. The cell invasion assay was performed using a modified Transwell chamber. PET inserts with 8 μm pores were pre-coated with 30 μl 20% Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA) diluted in RPMI-1640 media. A total of 100 μl cells ($1x10^6$/ml) (HXO-Rb44-GI-KAI1$^+$, HXO-Rb44-Gl-KAI1$^-$, HXO-Rb44-Gl; Y79-KAI1$^+$, Y79-KAI1$^-$, Y79) and 200 μl serum free RPMI-1640 media were added to the top chamber. A total of 500 μl RPMI 1640 media with 10% FBS was added to the bottom chamber. Each cell line was plated in three duplicate wells. Following 48 h incubation (37˚C, 5%CO_2), the invasive cells in the bottom chambers were counted using a light microscope in five randomly selected visual fields.

Statistical analysis. Values are presented as the mean ± standard deviation. Data was analyzed using the Student's *t*-test (Two groups comparisons), or one-way analysis of variance (ANOVA) test (multigroups comparisons) with *post hoc* contrasts by Student-Newman-Keuls test, and using Statistical Analysis Software (SAS 9.3 Institute, Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in KAI1 expression in human RB tissues. KAI1 mRNA and protein expression in human RB tissue was analyzed using RT-qPCR and western blot analysis. A ~2-fold decrease in KAI1 protein expression (Fig. 1A and B; P=0.0061) and ~4-fold decrease in KAI1 mRNA expression was observed in the invasive cells compared with the non-invasive cells (Fig. 1C; P<0.0001).

KAI1 overexpression in transduced RB cell lines. Expression of KAI1 in transduced HXO-Rb44-Gl and Y79 cells was analyzed using RT-qPCR, western blotting and immunofluorescence staining (Fig. 2). A >100-fold increase in KAI1 mRNA expression was observed in the KAI1$^+$ cell lines (Fig. 2A and B; P<0.0001). Positive KAI1 bands at 60 kDa were observed in the KAI1$^+$ cell lines and not in the KAI1$^-$ or control cells (Fig. 2C and D). In addition, KAI1 protein expression was observed in the cell membrane and nucleus of HXO-Rb44-GI-KAI1$^+$ cells using immunofluorescence staining but was not observed in the corresponding KAI$^-$ cells (Fig. 2E-G). These results are consistent with immunofluorescence staining patterns observed in previous studies (22).

Effect of KAI1 overexpression on cell growth. CCK-8 analysis was performed on HXO-Rb44-Gl cells and Y79 cells over 7 consecutive days. No significant difference in cell growth was observed between the KAI1 overexpressing and control cells (Fig. 3).

Effect of KAI1 on RB cell migration and invasion. The migration and invasion of transduced HXO-Rb44-Gl cells and Y79 cells was compared with control cells. The number of migratory cells in the HXO-Rb44-Gl-KAI1$^+$ group was significantly decreased compared with the HXO-Rb44-Gl-KAI1$^-$ and HXO-Rb44-Gl groups (Fig. 4A; P<0.0001). Similarly, the number of migrated cells in the Y79-KAI1$^+$ group was significantly decreased compared with the Y79-KAI1$^-$ and Y79 groups (Fig. 4B; P<0.0001).

A statistically significant reduction in the invasive ability of the cells was observed in the KAI1 and Y79 transduced cell lines compared with the respective control cell lines. The number of invasive cells was 11.65±4.85, 41.55±5.92 and 40.95±5.67 in the HXO-Rb44-Gl-KAI1$^+$, HXO-Rb44-Gl-KAI1$^-$ and HXO-Rb44-Gl cell lines, respectively (Fig. 4C; P<0.0001). The number of invasive cells was 10.20±3.20, 31.55±3.89 and 33.20±5.32 in the Y79-KAI1$^+$, Y79-KAI1$^-$ and Y79 cell lines, respectively (Fig. 4D; P<0.0001).

Discussion

Poor RB prognosis is primarily due to the occurrence of distant metastasis and organ infiltration (5). Developing novel methods of metastasis inhibition is an important aim of cancer research. The KAI1 gene is a known suppressor of tumor metastasis (23-25) and has been implicated in the regulation of cell adhesion, proliferation, motility, fusion, signaling and differentiation (26). To investigate the effect of KAI1 on RB migration and invasion, KAI1 expression in RB tissue was investigated in the present study. In addition, the effect of KAI1 overexpression on RB cell migration and invasion was examined.

KAI1 expression levels in RB tissues were evaluated, and KAI1 mRNA and protein were observed to be expressed at decreased levels in invasive RB tissue compared with non-invasive RB tissue. Consistent with the results of the present study, a previous report demonstrated a reduction in the expression of KAI1 protein in metastasized human RB samples (16). Similar results were reported in other types of tumor tissue, for example, increased KAI1 protein expression was observed in early stage colorectal cancer, but the expression level decreased as the cancer progressed into the later stages (27). Research on breast cancer demonstrated a 10-fold decrease in KAI1 mRNA expression in metastatic lesions compared with the primary tumors (28). Therefore, the reduction in KAI1 expression may be involved in the RB metastatic process.

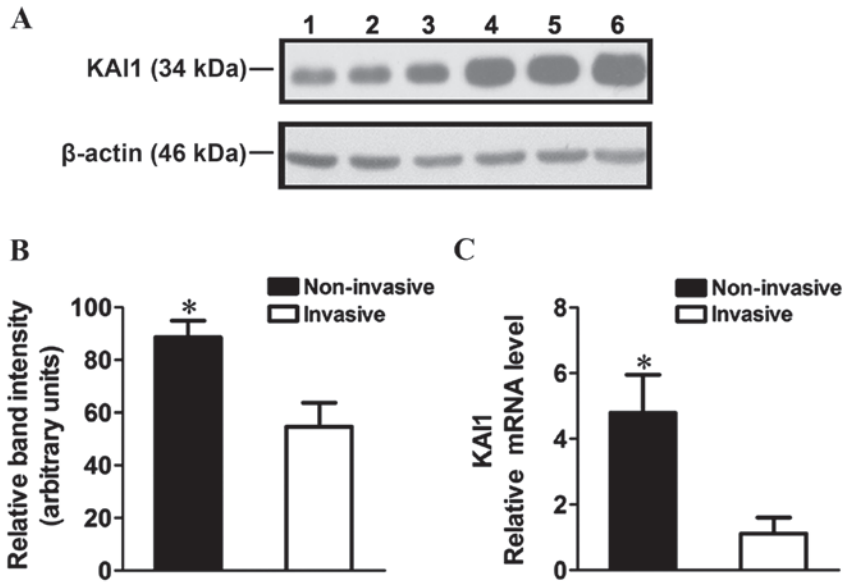

Figure 1. KAI1 expression in RB tissues. (A) Western blot analysis demonstrated decreased KAI1 protein expression in invasive RB samples (lanes 1-3) compared with non-invasive RB samples (lanes 4-6). (B) Relative band intensity of KAI1 protein in non-invasive RB tissue compared with invasive RB tissue, normalized to β-actin expression (*P<0.05). (C) Relative KAI1 mRNA expression level in non-invasive RB tissue compared with invasive RB tissue, normalized to GAPDH mRNA expression (*P<0.05). KAI1, cluster of differentiation 82; RB, retinoblastoma.

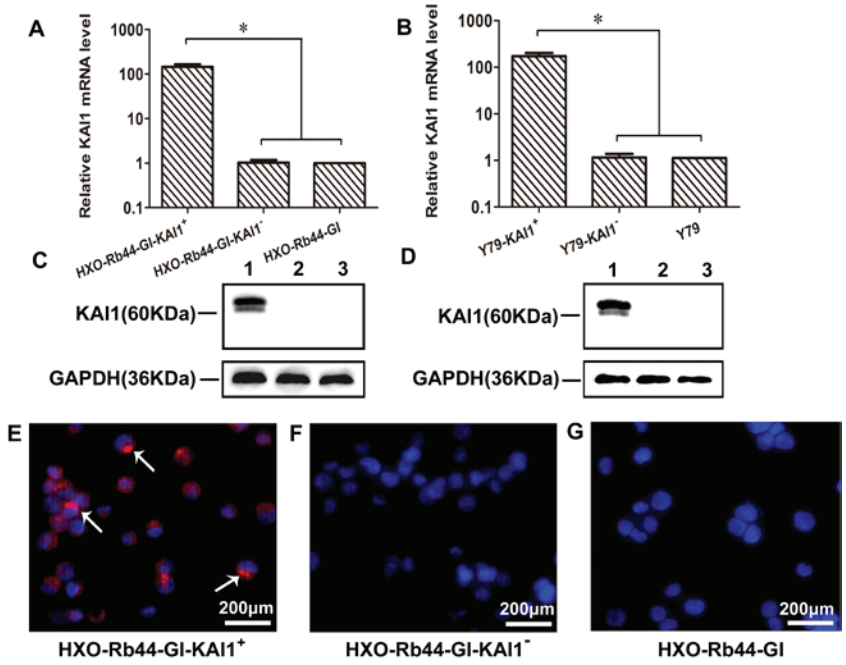

Figure 2. Overexpression of KAI1 in RB cell lines. (A) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated a significant increase in KAI1 mRNA expression in HXO-Rb44-Gl-KAI1+ cells compared with HXO-Rb44-Gl-KAI1- and HXO-Rb44-Gl cells (*P<0.05). (B) KAI1 mRNA expression in Y79-KAI1+ cells was significantly increased compared with Y79-KAI1- and Y79 cells (*P<0.05). (C) Western blot analysis demonstrated that KAI1 protein is overexpressed in HXO-Rb44-Gl-KAI1+ cells (lane 1) but undetectable in HXO-Rb44-Gl-KAI1- (lane 2) and HXO-Rb44-Gl cells (lane 3). KAI1-EGFP protein is the sum of the KAI1 protein and the EGFP protein, 60 kDa. (D) KAI1 protein was overexpressed in Y79-KAI1+ cells (lane 1) but undetectable in Y79-KAI1- (lane 2) and Y79 cells (lane 3). KAI1 expression and distribution was evaluated using immunofluorescence staining. KAI1 (red) staining by rhodamine, was mainly observed on the membrane and in the cytoplasm of the (E) HXO-Rb44-Gl-KAI1+ cells. (F) KAI1 staining was undetectable in the HXO-Rb44-Gl-KAI1- cells and (G) HXO-Rb44-Gl cells (magnification, x200). KAI1, cluster of differentiation 82; RB, retinoblastoma; EGFP, enhanced green fluorescent protein.

The results of the present study demonstrated that overexpression of KAI1 in HXO-Rb44-Gl cells and Y79 cells did not have any significant effect on cell growth, which is consistent with observations made in breast and other types of cancer (29,30). Increased levels of migration and invasion are associated with cancer metastasis, and previous research using the Boyden chamber assay indicated that pancreatic cancer cells infected with KAI1 demonstrate a reduced invasive ability (30). Similar observations were reported in studies in hepatocellular carcinoma and breast cancer (29,31). Consistent with the literature,

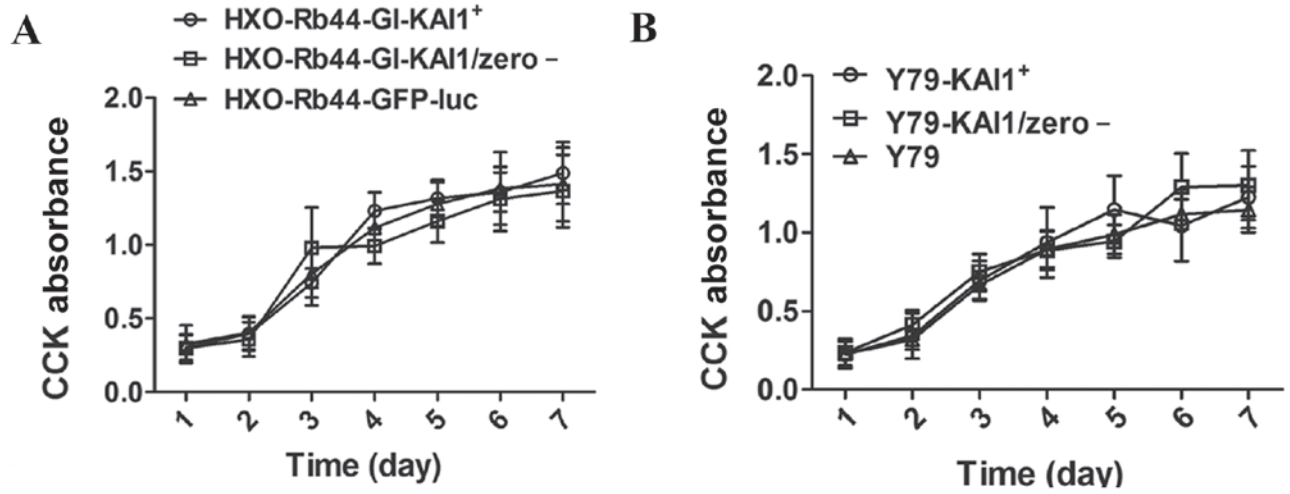

Figure 3. Growth curves of (A) HXO-Rb44-GI and (B) Y79 RB cells. No significant difference was observed between KAI1-overexpressing RB cells and the control cells. RB, retinoblastoma; KAI1, cluster of differentiation 82; CCK-8, cell counting kit-8.

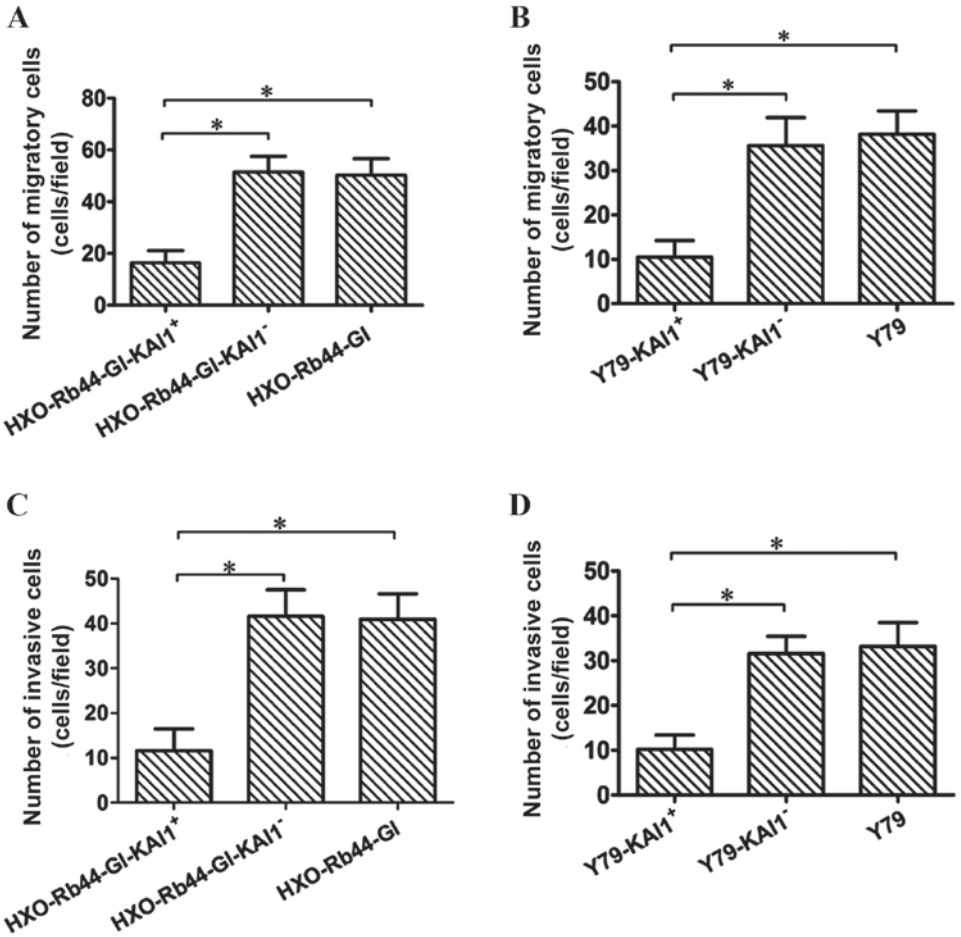

Figure 4. Cell migration and invasion assays. (A) The number of migratory cells in the HXO-Rb44-Gl-KAI1+ group was significantly reduced compared with the control groups (*P<0.05). (B) The number of migratory cells in the Y79-KAI1+ group was significantly reduced compared with the control groups (*P<0.05). (C) The number of invasive cells in the HXO-Rb44-Gl-KAI1+ group was significantly reduced compared with the control groups (*P<0.05). (D) The number of invasive cells in the Y79-KAI1+ group was significantly reduced compared with the control groups (*P<0.05). RB, retinoblastoma; KAI1, cluster of differentiation 82.

the results of the present study demonstrated that migration and invasion is significantly inhibited in HXO-Rb44-Gl-KAI1+ and Y79-KAI1+ cells compared with KAI1- RB cells, suggesting that KAI1 suppresses migration and invasion in RB.

However, the exact mechanism of KAI1-mediated cancer metastasis inhibition remains unclear. It has been reported that KAI1 may suppress tumor metastasis by linking to cell surface molecules, including tetraspanins, integrins, epidermal growth

factor receptor and protein kinase C (32,33). Additionally, KAI1 has been demonstrated to stabilize E-cadherin and β-catenin complexes in malignant cells, inhibiting tumor metastasis (34). Epithelial-mesenchymal transition (EMT) increases levels of cell migration, causing epithelial cells to lose epithelial characteristics and gain a mesenchymal phenotype (35). Previously, KAI1 has been demonstrated to cause certain EMT-associated genetic changes, including upregulation of cadherin 1 and catenin-α 1, and downregulation of hepatocyte growth factor and fibronectin 1 (22). In addition, upregulation of matrix metalloproteinases, and downregulation of TIMP metalloproteinase inhibitor 1 and SRC proto-oncogene, non-receptor tyrosine kinase are implicated in the inhibition of KAI1 in metastatic tumors (22). Therefore, KAI1 may suppress metastasis by regulating EMT, and regulating the migratory and invasive abilities of RB cells, however further research into the molecular mechanisms of KAI1-mediated metastasis inhibition in RB is required.

In conclusion, KAI1 may serve an essential role in the regulation of malignant cell migration and invasion in RB. The results of the present study may aid in the development of novel treatments to prevent and regulate RB metastasis.

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