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KIN enhances stem cell-like properties to promote chemoresistance in colorectal carcinoma

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ABSTRACT

Chemotherapy is widely used in colorectal cancer (CRC) treatment, especially in advanced stage patients. However, it is inevitable to develop chemoresistance. Recently, cancer cells acquired stem cell-like properties or cancer stem cells (CSC) were proved to attribute to chemoresistance. Here, we found that KIN protein was elevated in CRC cell lines and tissue specimens as compared to normal controls. Upregulation of KIN positively correlates with the metastatic status of CRC patients. Patients with high KIN expression showed poor prognosis and were with a short survival time. Overexpression of KIN enhanced, while silencing KIN impaired, chemoresistance to oxaliplatin (Ox) or 5-fluorouracil (5-FU) in CRC cell lines. Further investigation demonstrated that overexpression of KIN rendered CRC cells enriching CSC markers and CSC phenotype, and silencing KIN reduced CSC markers and CSC phenotype. Our findings suggest that the KIN level may be a suitable marker for predicting chemotherapy response in CRC, and silencing KIN plus chemotherapy may be a novel therapy for CRC treatment.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer related death worldwide, accounting for 1.2 million new cases and over 600,000 deaths each year [1]. The primary treatment for early CRC is surgery. However, chemotherapy is the preferred treatment for either reducing local recurrence or treating advanced/unresectable metastatic CRC [2,3]. Oxaliplatin and 5-fluorouracil (5-FU) are the first-line regimens for CRC chemotherapy. Oxaliplatin disrupts DNA replication and transcription by forming platinum–DNA adducts [4–6]. 5-FU inhibits activity of thymidylate synthase during DNA replication [7]. Chemotherapy prolonged the mean survival of CRC patients; however, the response rate of first-line chemotherapy is only near 50% [8] and resistance develops in nearly all patients. Thus, it is of great importance to study resistant mechanisms and to find therapies targeting resistance pathways.

Tumor-initiating cells or cancer stem cells (CSCs) have been prospectively identified in various kinds of tumors, such as prostate [9], breast [10], brain [11,12], head and neck [13] and colorectal cancer [14,15]. CD44 [16,17] and CD133 [18,19] are proposed to be the promising markers for CRC stem cells. Additional markers include CD166, ALDH1, CD24, CD26 and so on [20]. The pluripotency genes, such as Oct4, c-myc, Nanog, Klf-4 and so on, are considered as promising surrogate markers. Increasing body of evidence proved that CSCs account for chemoresistance [21–23].

KIN, also known as Kin17, was first identified by Angulo and colleagues in mice [24]. Then KIN was proved to be remarkably conserved during evolution and it was conserved among metazoans [25]. KIN encodes a zinc finger protein and forms nucleoplasmic foci resembling the proteins involved in DNA repair, replication and RNA splicing [25]. Indeed, KIN was proved to participate in DNA replication and was detected in complexes that mediate different types of nucleic acid transaction [26,27] and complexes of human spliceosome [28,29]. Certain tumor cell lines overproduce KIN and depletion of KIN increases the radiosensitivity of RKO cells [30]. Thus KIN is a potent target in treatment of cancer. However, the role of KIN in tumorigenesis remains largely unexplored.

In the present study, we found that KIN was significantly elevated in CRC and could be an independent prognosis factor. Overexpression of KIN decreased, while silencing KIN increased, sensitivity to oxaliplatin and 5-FU treatment. Further investigation found that upregulation of KIN enhanced stemness of CRC cells as indicated by the increased expression of stem cell markers, enhanced ability of tumor sphere formation and enriched subpopulation cells, and *vice versa*. Collectively, our finding

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supports the fact that elevated KIN accounts for chemoresistance in CRC and depletion of KIN could be a potent therapy for CRC treatment.

2. Materials and methods

2.1. Tissue specimens and ethics statement

For the use of clinical materials for research purposes, 12 fresh CRC and matched adjacent normal tissues and 74 paraffin-embedded, archived CRC specimens were collected from the First Affiliated Hospital, Sun Yat-sen University with prior written informed consents from the patients and approval from the Institutional Research Ethics Committees of the hospital ethics Committee. All samples were histopathologically and clinically diagnosed. The clinical information was summarized in Supplemental Table 1.

2.2. Immunohistochemical analysis (IHC)

Immunohistochemical analysis was performed according to a previous report [31]. Briefly, paraffin-embedded specimens were cut into 4-μm sections and baked at 60 °C for 2 h, followed by deparaffinization with xylene and rehydrated. The sections were submerged into EDTA antigenic retrieval buffer and microwaved for antigenic retrieval, after which they were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, followed by incubation with 1% bovine serum albumin to block nonspecific binding. Sections were incubated with rabbit anti-KIN (1:50, Santa Cruz) overnight at 4 °C. Normal goat serum was used as a negative control. After washing, tissue sections were treated with biotinylated anti-rabbit secondary antibody (Invitrogen), followed by further incubation with streptavidin–horseradish peroxidase complex (Invitrogen). Tissue sections were then immersed in DAB (3.3′-diaminobenzidine) and counterstained with 10% Mayer's hematoxylin, dehydrated and mounted. IHC staining for protein expression in tumor lesions and normal tissues was quantitatively analyzed with the AxioVision Rel.4.6 computerized image analysis system assisted with an automatic measurement program (Carl Zeiss, Oberkochen, Germany).

2.3. Cell culture, plasmids and stable cell line establishment

Colorectal cancer (CRC) cell lines, including SW480, LoVo, DLD1, HT29, HCT116, RKO, were purchased from ATCC and were cultured in RPMI 1640 medium (Gibco, Life Technology) supplemented with 10% FBS (Gibco, Life Technology) in a humidified 5% CO_2 atmosphere at 37 °C.

To overexpress KIN, the coding sequence of human KIN was amplified from cDNA by PCR and subcloned into the Bgl II/Xho I sites of pMSCV-retro-puro vector. And the primer sequences are: forward, 5′-GCCAGATCTGCCATGGGGAAGTCGGATTTTC-3′, and reverse, 5′- GCCCTCGAGTCAGGCAAGTTTAGAAATGTC-3′. To silence endogenous KIN, a shRNA oligonucleotides were designed and cloned into the pSuper-retro-puro vector to generate pSuper-retro-KIN-RNAi, and the target sequence is CAGCAGTT TATGGAT TATT. Retroviral production and infection were performed as previously described [32]. Stable cell lines expressing KIN or KIN RNAi were selected by treatment for 10 days with 0.5 μg/ml puromycin beginning 48 h after infection.

2.4. Cell lysate preparation and Western blot analysis

Cells were washed with ice-code PBS and then harvested in sample buffer (125 mM Tris–HCl, pH 6.8, 6% SDS, 20% glycerol) with antiprotease cocktail. Lysates were stored frozen at −80 °C until use. 20–40 μg of total protein was subjected to SDS–PAGE electrophoresis and transferred onto PVDF membranes (Roch Applied Science). Membranes were blocked with 5% dry milk in TBST and immunoblotted with primary antibodies as follows: anti-KIN (Santa Cruz), anti-c-Myc (Cell Signaling Technology), anti-CD44, anti-Nanog (Abcam). α-Tubulin (Sigma Aldrich) antibody was used as loading control. HRP conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and enhanced chemiluminescence (Pierce) were used to detect the protein bands.

2.5. MTT assay

MTT assay was performed according to the previous report [33]. Briefly, firstly, seed cells at 5000 cells per well in 200 μl medium and keep it overnight. The next day, change the medium with solutions of oxaliplatin at indicated concentrations. After 48 h of drug incubation, 20 μl of MTT was added to each well and incubated for 4 h. Then remove the supernatant and add 100 μl of dimethyl sulfoxide to dissolve the precipitate. Absorbance was measured at 570 nm and plotted against the drug dose.

2.6. Colony formation assay

5×10^3 cells were plated into 6-well plates and kept overnight to allow attachment. The next day change media with solutions of oxaliplatin (Ox, 10 μM) or 5-FU (10 μg/ml). 10 days later cells were fixed with ice cold methanol for 10 min and stained with 0.1% crystal violet for another 10 min. Representative images were scanned by GS-800™ Calibrated Densitometer (Bio-RAD).

2.7. Flow cytometry analysis of cell subpopulation

Cells were trypsinized and re-suspended at 1×10^6 cells/ml in RPMI 1640 containing 2% fetal bovine serum (FBS) and then pre-incubated at 37 °C for 30 min with or without 100 μM verapamil (Sigma Aldrich). The cells were subsequently incubated for 90 min with 5 μg/ml Hoechst 33342 (Sigma Aldrich) at 37 °C. Finally the cells were incubated on ice for 10 min and washed with ice-cold PBS before flow cytometry analysis. Data were analyzed by Summit5.2 (Beckman Coulter, Indianapolis, IN).

2.8. Sphere formation assays

2000 cells were plated in 6-well ultra low cluster plates (Corning). Cells were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 20 ng/ml of EGF, and 20 ng/ml of bFGF (PeproTech), 0.4% bovine serum albumin (BSA) (Sigma), and 5 μg/ml insulin. Spheres were photographed and counted after being cultured for 1 week.

2.9. RNA extraction, reverse transcription (RT) and real-time PCR

Total RNA from cultured cells was extracted using the Trizol reagent (Invitrogen) as the manufacturer instructed. cDNAs were amplified and quantified in CFX96™ Real-Time PCR Detection System (Bio-RAD) using FastStart SYBR Green Master (Roche Applied Science). The primers were selected as follows: CD44, forward, 5′-CGGACACCATGGACAAGTTT-3′, and reverse, 5′-CGTGGAATACAC CTGCA AAG-3′; c-Myc, forward, 5′-TCAAGAG GCGAACACACAAC-3′, and reverse, 5′-GGCCTTTTCATTGTTTTCCA-3′; Nanog, forward, 5′-GATTTGTGGGCCTGAAG AAA-3′, and reverse, 5′-CAGGGCTGTCCT GAATAAGC-3′; Oct4, forward, 5′-GTGGAGGAAGCTGACAACAA-3′, and reverse, 5′-TCTCCAGGTTGCCTCTC ACT-3′; ABCG2, forward, 5′- CTGAGATCCTGAGCCTTTGG-3′, and reverse, 5′-AAGCCATTGGTG TTTCCTTG-3′; Expression data were normalized to the geometric

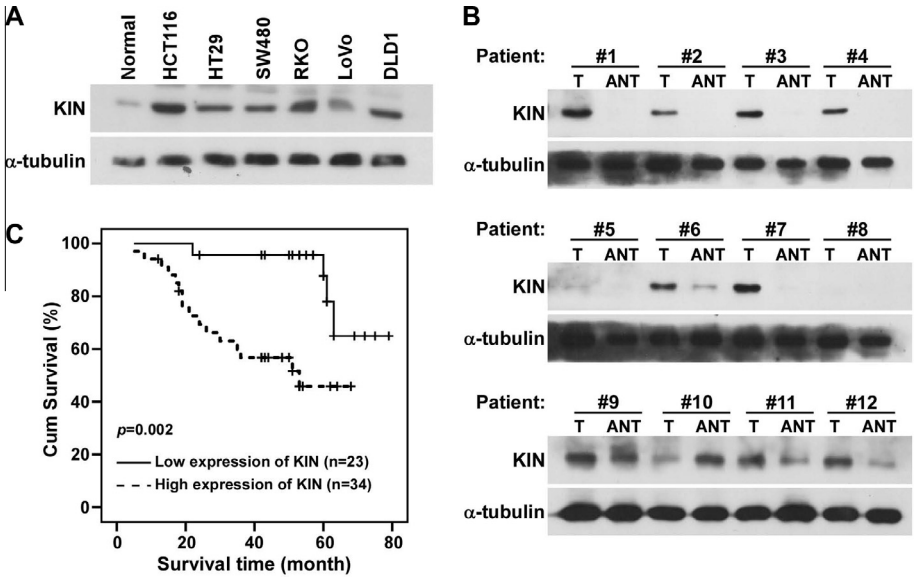

Fig. 1. KIN is elevated in CRC cell lines and clinical specimens. (A) Western blotting analysis of KIN in CRC cell lines and normal colon epithelial cells. α-Tubulin was used as loading control. (B) Western blotting analysis of KIN in paired tissues freshly collected from patients. α-Tubulin was used as loading control. (C) Kaplan-Meier curves with univariate analyses (log-rank) for patients with low KIN expressing versus high KIN expressing tumors.

mean of housekeeping gene *GAPDH* (forward: 5′-ACCACAGTCCATG CCATCAC-3′ and reverse: 5′-TCCACCACCCTG TTGCTGTA-3′) to control the variability in expression levels and calculated as $2^{-[(C_t \text{ of gene})-(C_t \text{ of GAPDH})]}$, where C_t represents the threshold cycle for each transcript. Three biological replicates, resulting from three different RNA extractions were used for quantification analysis and

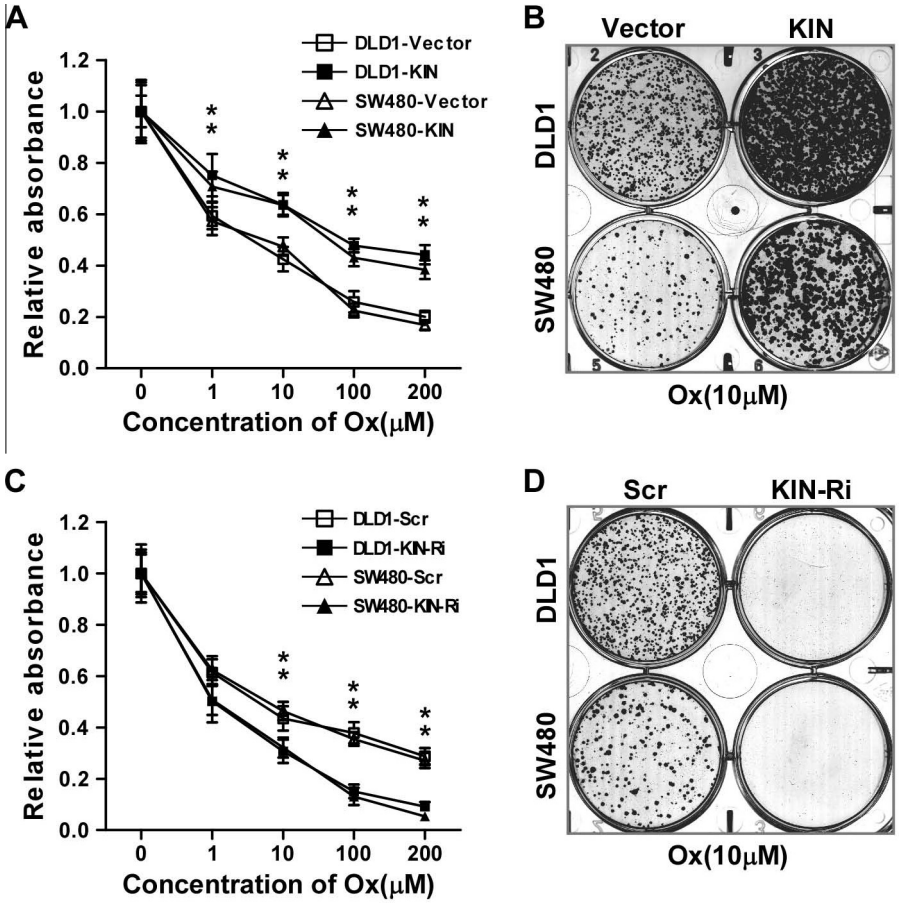

Fig. 2. Expression of KIN associates with chemoresistance in CRC cells. (A) Overexpression of KIN significantly decreased the growth-inhibitory effect of oxaliplatin (Ox) in indicated cells, as measured by MTT assay. (B) Overexpression of KIN formed more colonies than vector cells in the presence of oxaliplatin (Ox, 10 μM). (C) Knockdown of KIN significantly increased the growth-inhibitory effect of oxaliplatin (Ox) in indicated cells, as measured by MTT assay. (D) Knockdown of KIN formed fewer colonies than vector cells in the presence of oxaliplatin (Ox, 10 μM).

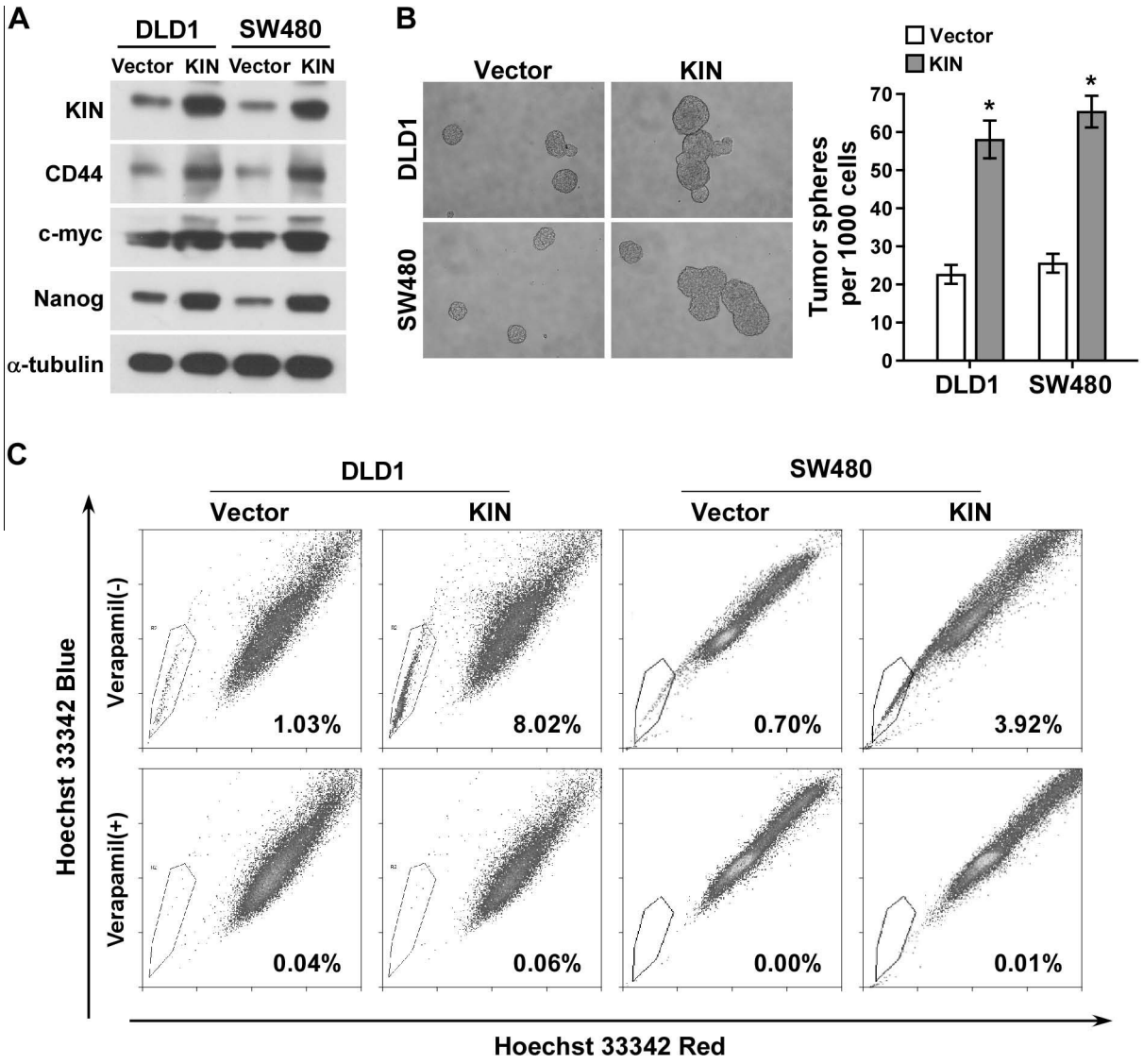

Fig. 3. Overexpression of KIN enhances stem cell-like properties of CRC cells. (A) Overexpression of KIN upregulated stem cell markers in indicated cells, as indicated by Western blotting. (B) Overexpression of KIN formed more tumor spheres than vector cells. Representative micrographs (200×, left panel) and quantification (right panel) of tumor spheres. (C) Hoechst 33342 dye exclusion assay showing that overexpression of KIN increased the side population (SP) cells in the indicated cells, as measured by flow cytometry analysis.

three technical replicates were analyzed for each biological replicate.

3. Results

3.1. KIN is elevated in CRC cell lines and clinical specimens and associates with poor prognosis and metastatic status of CRC patients

In the very beginning, we detected the expression of KIN in both CRC cell lines and freshly collected paired tissues. As shown in Fig. 1A, KIN was significantly overexpressed in CRC cell lines compared to the normal colorectal epithelia cells. And KIN was also dramatically elevated in 12 CRC samples as compared to matched adjacent normal tissues in fresh tissues. Further we performed IHC staining in a cohort of 74 paraffin-embedded, archived CRC specimens. The IHC result showed that KIN was differently upregulated in CRC tissues as compared with adjacent normal tissues (Supplemental Fig. 1). What is more, statistical analysis revealed that the expression of KIN associated with metastatic status (Supplemental Table 2) and survival time of CRC patients. Patients with high expression of KIN showed significantly shorter survival time than the patients with low expression of KIN as determined by the Kaplan–Meier and log-rank tests for survival analysis ($P = 0.002$, Fig. 1C).

3.2. Expression of KIN associates with chemotherapy sensitivity of CRC cells

Chemotherapy is the major adjunctive treatment for early stage CRC, and main therapeutic strategy for metastatic CRC. Thus, we determined the effects of KIN expression on chemotherapy sensitivity or chemoresistance. As expected, we found that upregulation of KIN significantly enhanced the chemoresistant activity to oxaliplatin treatment of DLD1 and SW480 cells compared to the vector cells (Fig. 2A), and KIN overexpressing cells formed more colonies under the treatment of oxaliplatin (Fig. 2B) or 5-FU (Supplemental Fig. 2A). Nevertheless, downregulation of KIN in CRC cells increased their sensitivity to chemotherapy (Fig. 2C) and impaired their ability of form colonies under the treatment of oxaliplatin

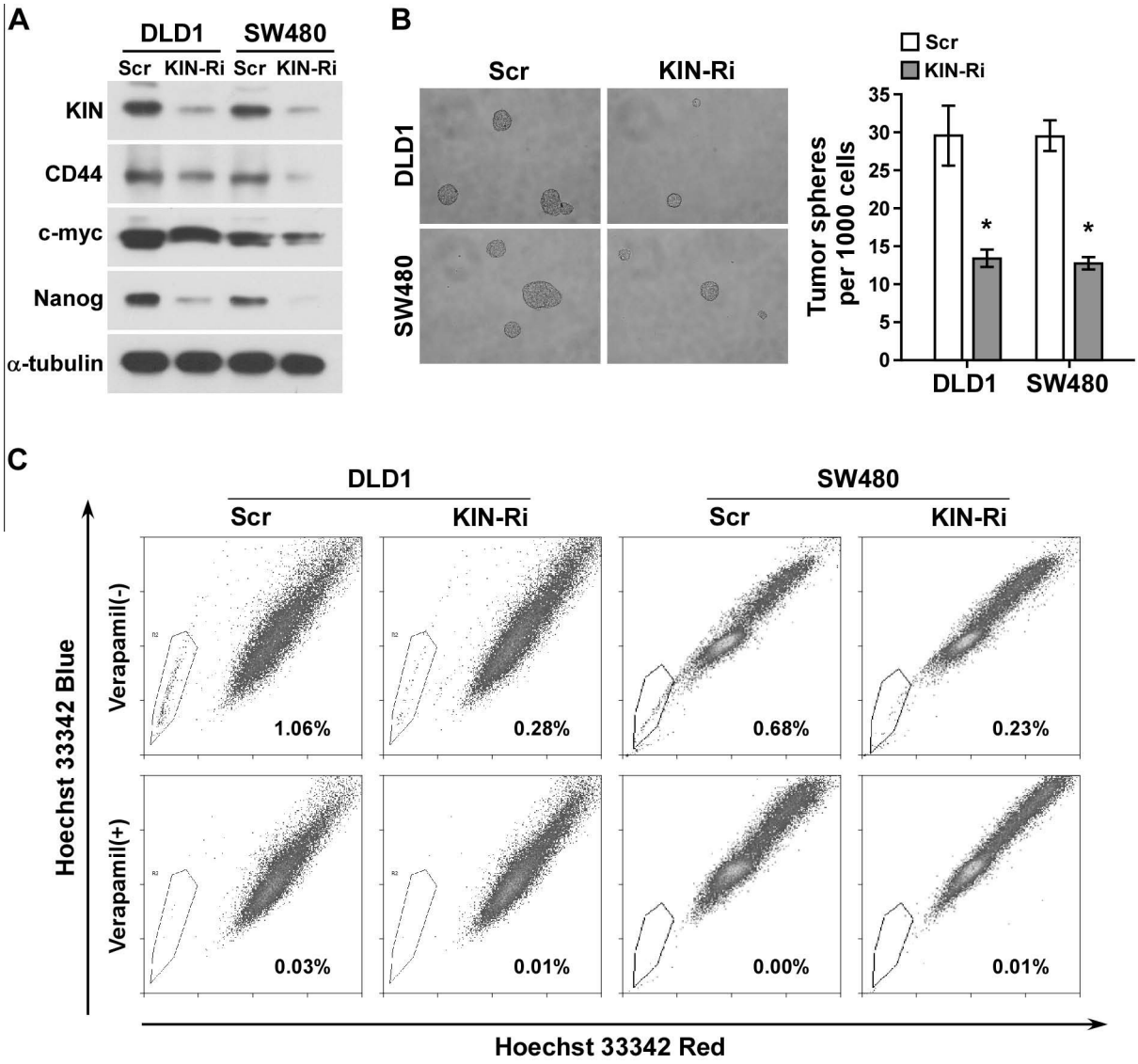

Fig. 4. Silencing KIN impairs stem cell-like properties of CRC cells. (A) Silencing KIN downregulated stem cell markers in indicated cells, as indicated by Western blotting. (B) Silencing KIN formed less tumor spheres than vector cells. Representative micrographs (200×, left panel) and quantification (right panel) of tumor spheres. (C) Hoechst 33342 dye exclusion assay showing that silencing KIN increased the side population (SP) cells in the indicated cells, as measured by flow cytometry analysis.

(Fig. 2D) or 5-FU (Supplemental Fig. 2B). Here, we draw that the expression of KIN correlates positively with chemotherapy sensitivity of CRC cells. Elevated KIN promotes chemoresistance of CRC cells.

3.3. Upregulation of KIN enhances stem cell-like properties of CRC cells

It has been evident that acquiring stem cell-like properties or increasing population of cancer stem cells enhanced chemoresistance in different kinds of tumors. We tested whether KIN enhanced stem cell-like properties of CRC could promote chemoresistance. We found that overexpression of KIN led to upregulation of stem cell markers, such as CD44 c-myc, Nanog and so on, in both protein (Fig. 3A) and mRNA (Supplemental Fig. 3A) levels in both CRC cell lines. Tumor sphere culture assay revealed that DLD1-KIN and SW480-KIN cells formed more and larger spheres as compared to vector control cells (Fig. 3B). And also, overexpression of KIN dramatically increased the percentage of subpopulation cells in both cell lines, 8.02% of DLD1-KIN vs. 1.03% of DLD1-vector cells, and 3.92% of SW480-KIN vs. 0.70% of SW480-vector cells.

3.4. Silencing KIN impairs stem cell-like properties of CRC cells

To confirm that KIN enhanced stem cell-like properties of CRC cells, we further silenced endogenous KIN expression by RNAi. As shown, silencing KIN significantly decreased the protein expression (Fig. 4A) and mRNA expression (Supplemental Fig. 3B) of stem cell markers. Tumor sphere culture assay showed that KIN-RNAi cells formed fewer and smaller spheres as compared to control cells (Fig. 4B). Flow cytometry analysis evidences that knockdown of KIN dramatically decreased the percentage of subpopulation cells in both cell lines.

4. Discussion

Human KIN is located on chromosome 10 at p15-p14 and encodes a 45KD zinc finger protein. The expression of KIN was widely detected in human tissues and organs while being cloned. KIN was ubiquitously expressed, and it showed higher expression level in heart, skeletal muscle and testis comparing to other tested tissues [25]. However, the expression of KIN in the disease of

cancer is seldom investigated. Recently, Zeng et al. proved that KIN was significantly upregulated in breast cancer [34]. Kannouche et al. found that SW480 cells showed much higher expression of KIN than non-tumorigenic cells or primary human fibroblasts [25], which is a sign of upregulation of KIN in CRC. Indeed, here we showed that KIN was highly expressed in CRC cell lines and clinical specimens as compared to normal controls. And we also found that KIN was an independent prognosis factor for CRC. Patients with higher KIN showed significantly shorter survival time ($p < 0.001$).

Chemotherapy is an important component of CRC treatment, especially for metastatic cancers. However, the response rate is only near 50% even for the first-line chemotherapy, and nearly all patients develop chemoresistance finally [8]. Thus, new strategies should be applied to increase the sensitivity to toxic chemotherapy or to deduce chemoresistance. In our current study, we found that the level of KIN positive correlated with chemotherapy response. Overexpression of KIN enhanced, while silencing KIN impaired, chemoresistance to oxaliplatin (Ox) or 5-fluorouracil (5-FU) in CRC cell lines. These findings indicated that KIN is a novel target of CRC treatment and depletion of KIN in CRC would increase sensitivity to chemotherapy.

Cancer stem cells (CSCs) were attributed to chemoresistance in different types of tumors [21–23]. The mechanisms that regulate cell cycle and promote DNA damage repair can protect CSCs form DNA damage radiation and chemotherapeutics, particularly cytotoxic drugs targeting tumor cell DNA [35]. This could be the mechanism for KIN mediated chemoresistance that was observed in our study. Biard and colleagues reported that ectopic expression of KIN induced a tremendous decrease in cell proliferation [36]. Kannouche et al. also found that overexpression of KIN protein inhibited cell cycle progression into S phase [37]. KIN also appears to play a pivotal role in DNA repair. UVC irradiation induces the expression and the nucleoplasmic redistribution of KIN, indicating a link between KIN and DNA damage response [25]. KIN is also induced in response to other DNA damage inducers, such as γ-irradiation [38] and MMC [39]. In breast cancer, silencing KIN reduced DNA damage repair [34].

All in all, in this present study we reported for the first time that KIN was dramatically elevated in CRC. Upregulation of KIN leads to poor prognosis and a short overall survival time in CRC patients. KIN promotes chemoresistance by enhancing stemness of CRC cells. These results indicated that depletion of KIN could be a novel strategy for CRC treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.057.

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