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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.

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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

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