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Research paper

Identification of PRKDC (Protein Kinase, DNA-Activated, Catalytic Polypeptide) as an essential gene for colorectal cancer (CRCs) cells

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ABSTRACT

Oncogene and non-oncogene addictions describe the phenomenon that tumor cells become reliant on certain genes for maintenance of malignancy. Reversal of these mutations profoundly affects tumor growth and survival, providing a fundamental rationale for development of targeted cancer therapy. However, inadequate knowledge on cancer signaling networks and lack of potential drug targets limited its clinical application. A screen was conducted using a custom small interfering RNA (siRNA) library in colorectal cancer (CRC). Transient knockdown followed by cell proliferation assays were performed to validate the essentiality of PRKDC (Protein Kinase, DNA-Activated, Catalytic Polypeptide) in CRC. Western blot analysis was performed to examine the mechanism by which PRKDC confers selective survival advantage in CRC cells. Inducible knockdown and overexpression cell lines were introduced into nude mice to assess PRKDC dependency of CRC cells *in vivo*. PRKDC expression level in patient samples and overall survival of patients with low or high PRKDC expression were analyzed. Transient knockdown of PRKDC reduced cell proliferation/survival in HCT116 and DLD1, but not FHC cells. PRKDC down-regulation induced apoptosis partially through inhibiting AKT activation, and sensitized HCT116 cells to chemotherapeutic agents interfering with DNA replication. Inducible knockdown of PRKDC inhibited tumor growth *in vivo*. PRKDC was up-regulated in cancerous tissues compared with normal tissues. Patients with high PRKDC expression showed poorer overall survival. PRKDC is an essential gene required for CRC cell proliferation/survival, which may represent as a potential prognostic biomarker and an ideal therapeutic target for CRC.

1. Introduction

It is widely accepted that cancer is a genetic disease that evolves through a multistage process that can extend over decades (Weinstein and Joe, 2008). This process is driven by accumulation of a series of genetic alterations involving the amplification, and/or overexpression of key oncogenes, gain-of-function mutation as well as the loss-of-function mutation, deletion, and/or epigenetic abnormalities (Luo et al., 2009). These mutational events contribute to the acquisition of a set of phenotypic traits including limitless cell proliferation potential, self-sufficiency in growth signals, resistance to apoptotic cues, sustained angiogenesis and evasion from immune surveillance, *etc.* (Luo et al., 2009). Despite the extensive disruption of genome, there are several cases in both experimental system and in patients where the restoration of only one or a few of these abnormalities can profoundly inhibit cancer cell growth (Weinstein and Joe, 2006). Oncogene addiction describes this phenomenon that tumor cells become reliant on one or a few genes for maintenance of the malignant phenotype (Weinstein, 2002; Weinstein and Joe, 2006). This concept can be extended to non-oncogenes based on the knowledge that the tumorigenic state depends on the activities of a diverse array of genes and pathways, many of which are not inherently oncogenic (Luo et al., 2009). Since cancer cells harbor very different intracellular circuitry compared to normal cells that leads to differential dependency on particular genes or pathways, oncogene and non-oncogene addiction have provided a foundational rationale for the development of targeted anti-cancer therapy (Weinstein and Joe, 2006). However, the clinical applications employing these concepts are still limited due to inadequate knowledge

Abbreviation: siRNA, small interfering RNA; CRC, colorectal cancer; PRKDC, Protein Kinase, DNA-Activated, Catalytic Polypeptide.

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about the complex cancer cell signal network. Therefore, there is an urgent need for identification of essential genes for cancer cells and elucidation of the underlying mechanism.

Cancer genome sequencing efforts have led to the identification of novel genetic mutations occurring at varying frequencies in many types of malignancy, including colorectal cancer (CRC) (Wood et al., 2007; Pleasance et al., 2010; Stratton, 2011). Frequently mutated genes are informally referred to as driver alterations or candidate cancer genes (CAN-genes), whereas those less frequently mutated are considered random mutational events or passenger mutations that do not directly contribute to tumorigenesis (Stratton, 2011). Initial efforts to sequence the CRC genome detected 151 highly mutated candidate genes and approximately 700 mutations occurring at a lower frequency (Wood et al., 2007). Those CAN-genes represent the potential driver genetic alterations directly involved in CRC tumorigenesis.

Advances in RNA interference (RNAi) technologies have facilitated the dissection of biological processes and cancer-associated phenotypes (Sims et al., 2011). Compared to whole-genome screening, RNAi screening focusing on specific gene sets obtained from cancer genome sequencing data represent a more efficient way for identifying important alterations involved in a specific aspect of cancer biology. In this study, we conducted a targeted RNAi screen using custom siRNA library focusing on the CAN-genes to identify those alterations that cause oncogene or non-oncogene addiction in CRC. PRKDC was identified as an essential gene for CRC cell growth/survival both *in vitro* and *in vivo* partially through its regulation of AKT activation. Therefore, PRKDC may represent a novel therapeutic target for intervention of CRC.

2. Materials and methods

2.1. siRNA screening and data analysis

Pooled siGENOME siRNAs (Dharmacon, Lafayette, CO, USA) were transfected into 1×10^5 cells using RNAiMAX per manufacturer's instructions 24 h after seeding the cells. Briefly, siRNAs and RNAiMAX were diluted in OptiMEM medium and plated onto cells. 48 h later, transfected cells were subjected to CellTiter-Glo® assay. Unsupervised hierarchical cluster analysis was performed on log2 ratios within all the cell lines.

2.2. Cell culture, patient samples and chemicals

Human colon cancer cell lines (HCT116, DLD-1, RKO *etc.*), normal cell lines and 293FT cells (ATCC, Manassas, VA, USA) were cultured in basal medium supplemented with 10% serum at 37 °C and 5% CO_2. FHC cell line is cultured in basal medium (Cat# 30-2006) supplemented with 10 mM HEPES, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, fetal bovine serum 10%. 5-fluouracil, oxaliplatin, taxotere, doxorubicin, and cisplatin were obtained from Sigma (St. Louis, MO, USA). Akt inhibitor IV was obtained from Santa Cruz (Dallas, TC, USA) and was used at 2 μM concentration. All the cancer samples and normal tissues were retrieved from The Central Hospital of Zaozhuang Mining Group of Shandong. This study was approved by The Central Hospital of Zaozhuang Mining Group of Shandong and written informed consent was obtained from all patients.

2.3. Chemo-sensitivity assay

Cells were seeded at a density of 5×10^3 cells/well in 96-well microtiter plates and allowed to attach overnight. Chemo drugs were then added and cultured for an additional 72 h. Cell viability was assessed using CellTiter-Glo® assay (Promega, Madison, USA). Each value was normalized to cells treated with DMSO and the IC_{50} values are calculated using Graphpad Prism software.

2.4. Plasmids and transfections

PRKDC cDNA (RG235406) and 3 individual siRNAs (SR303748) targeting PRKDC were obtained from Origene (Rockville, MD, USA). 1036 pcDNA3 Myr HA Akt1 was a gift from William Sellers (Addgene plasmid #9008). Tet-pLKO-puro was a gift from Dmitri Wiederschain (Addgene plasmid #21915). siRNAs were transfected with Lipofectamine RNAi Max reagent (Invitrogen, Grand Island, NY, USA) as per manufacturer's protocol. cDNA transfections were performed with Lipofectamine LTX reagent (Invitrogen) as per manufacturer's protocol.

2.5. Viral transductions and stable selections

For lentivirus production, 1 μg of plasmid together with 1 μg of helper plasmids (0.4 μg pMD2G and 0.6 μg psPAX2) were transfected into 293FT cells with Effectene reagent (Qiagen, Valencia, CA, USA). Viral supernatants were collected 48 h after transfections and cleared through a 0.45-μm filter. Cells were infected with viral supernatants containing 4 μg/ml polybrene (Sigma, St. Louis, MO, USA) and selected with puromycin for 7 days.

2.6. Caspase 3/7 activity

Control or miR-425-5p transfected cells were treated with vehicle control or chemo drugs for 72 h and subjected to Caspase-Glo 3/7 assay according to the manufacturer's instructions in 96-well plates (Promega, Madison, USA).

2.7. Immunoblot analysis

Total cell lysates were prepared by harvesting cells in Laemmli SDS reducing buffer (50 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol), boiled and resolved on an 8% to 10% polyacrylamide gel, and transferred to polyvinylidinefluoride. Antibodies against PRKDC (Abcam, Cambridge, USA), Phospho-Akt (Ser473, Cell Signaling, Danvers, MA, USA), total Akt (Cell Signaling), Cleaved PARP (Cell Signaling) and β-actin (Sigma-Aldrich) were used. The blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Dallas, USA) at a dilution of 1:5000 and detected with Super Signal West Pico or Femto Chemiluminescent Substrate Kit (Thermo Scientific, Grand Island, USA).

2.8. Xenograft experiments

All animal experiments were approved by Institutional Animal Care and Use Committee of National Cancer Center. HCT116 cells expressing vector control or shRNA targeting PRKDC with or without PRKDC overexpression (3×10^6 cells/injection) were subcutaneously injected into both flanks of 6 weeks old female nude mice ($n = 5$/group). Doxycycline (30 mg/kg, Sigma) was injected i.p. 6 days post implantation. Tumor volumes were measured using caliper and determined by a formula [volume = (length × width2) / 2] from day 8 to day 26 post implantation. The results were expressed as mean tumor volumes with SD.

2.9. Statistical analysis

Quantitative data are expressed as mean ± SD. Statistical significance was assessed by the two tailed Student t test. Differences were considered to be significant when $P < 0.05$. Long-rank test was performed to calculate P-value for survival curves.