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RAPID COMMUNICATION

Crotonylation of PRKACA enhances PKA activity and promotes colorectal cancer development via the PKA-FAK-AKT pathway

Colorectal cancer (CRC) is a common cancer with high morbidity and mortality.[1] Post-translational modification (PTM) of protein plays an important role in the pathogenesis of CRC. Lysine crotonylation (Kcr) is an important type of PTM and has been proved evolutionarily conserved in eukaryotic cells from a wide range of species.[2] However, the role of protein Kcr in the progression of CRC is unclear.

We first carried out global crotonylome study in human CRC tissues and paired adjacent paracancerous tissues using label free quantitative Kcr enrichment technique and quantitative proteomic analysis based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. S1A). All Kcr sites on proteins were identified, some of them had quantitative information (Table S1). After normalizing the protein quantitative group to remove the effect of protein expression on the modification, we found that the Kcr modification levels of most sites were upregulated (Fig. 1A and Table S2). We identified the characteristic motifs from the upstream and downstream of the crotonylated lysine residue sites and the subcellular distribution of crotonylated proteins (Fig. S1B, C). GO classification showed that the proteins with Kcr were involved in a variety of biological processes, including metabolic processes, cell junction, catalytic activity, etc (Fig. S1D, E). For further hierarchical clustering based on protein functional classification, we performed GO enrichment for Q1 to Q4, according to their differential multiples (Fig. S1F). The response to cAMP pathway based on GO biological process enrichment and KEGG enrichment attracted our attention (Fig. S1G). Consistent with this result, KEGG enrichment also proposed the cAMP signaling pathway, and the Kcr of the holoenzyme of protein kinase A (PKA) at the center of the pathway was upregulated (Fig. S1H). As shown in the protein–protein interaction network of protein kinase cAMP-dependent catalytic-alpha (PRKACA) (Fig. 1B), nearly half of the proteins closely related to PRKACA were regulatory subunits of PKA, suggesting that Kcr of PRKACA may play a role in the interaction of PRKACA and the regulatory subunits. Therefore, we focused on PRKACA to study the biological effects of Kcr in CRC.

Compared with normal colon cell line (NCM460), PRKACA Kcr increased in all three CRC cell lines (HCT116, SW480, and SW620) (Fig. S2A). To determine the crotonyltranferase and decrotonylase of PRKACA, we co-transfected candidates in HCT116 cells. Results showed that CREB-binding protein (CBP) mainly promoted the Kcr of PRKACA (Fig. S2B) and SIRT3 could effectively reduce the Kcr of PRKACA (Fig. S3A). The interaction between CBP or SIRT3 and PRKACA was also verified at the endogenous level (Fig. S2C, S3B). In HCT116 cells, knocking down endogenous CBP or SIRT3 by siRNA (small interfering RNA) effectively reversed the Kcr of transfected PRKACA (Fig. S2D, S3C). Furthermore, CBP inhibitor A485 or SIRT3 inhibitor 3-TYP had the same effect in a dose-dependent manner in HCT116, SW480, and SW620 cells (Fig. S2E, S3D). In HCT116 cells, effective downregulation of SIRT3 on Kcr and upregulation of CBP on Kcr could counteract each other by co-transfection (Fig. 1C). In addition, we discovered that the level of SIRT3 in CRC tissues was significantly lower than that in the adjacent tissues (Fig. S4A–C) and overexpression of SIRT3 could significantly reduce the proliferation rate, colony formation, migration, and invasion of HCT116 cells (Fig. S4D–G). To sum up, CBP is crotonyltranferase of PRKACA and SIRT3 is decrotonylase of PRKACA. SIRT3 may has a potential effect on the development of CRC.

The LC-MS/MS analysis showed that lysine (K) residue 84 was the main crotonylation site of PRKACA (Fig. S5A) and K84 in PRKACA was evolutionarily conserved in different species (Fig. S5B). PKA exists in the form of tetramer consisting of two inactive catalytic subunits (C) and two

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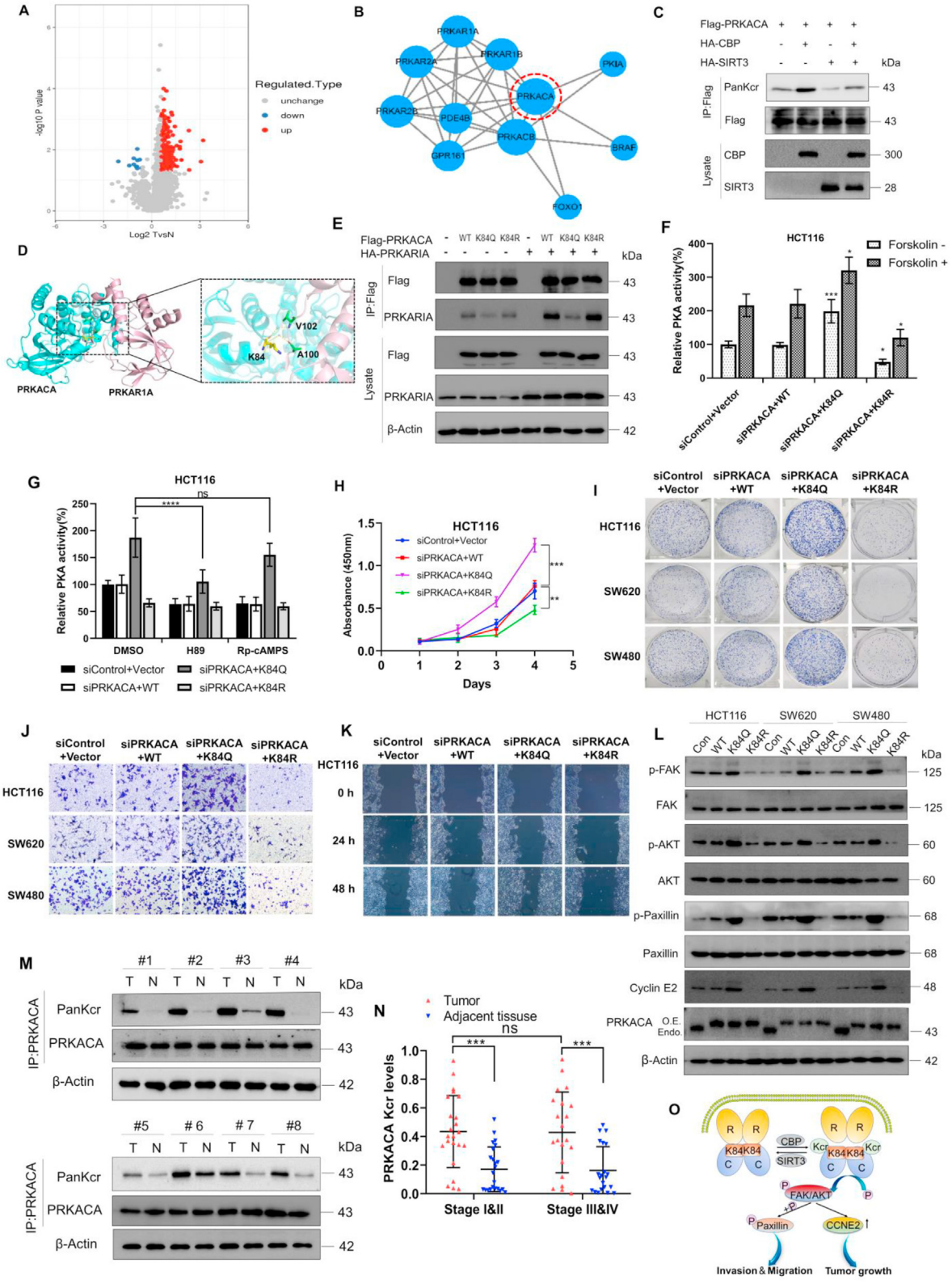

Figure 1 Crotonylation of PRKACA enhances PKA activity and promotes colorectal cancer development via the PKA-FAK-AKT pathway. **(A)** Scatterplot showing the numbers of differentially modified sites in comparison groups. The horizontal axis is the value of difference multiple after logarithmic conversion of Log2, and the vertical axis is the −Log10 logarithmic conversion of *P*-values for the significance test of difference. **(B)** PRKACA Kcr-centric protein−protein interaction network based on STRING database. **(C)** HCT116 cells were transfected with indicated plasmids, and cell lysates were harvested for IP and immunoblotting. **(D)** Molecular modeling prepared using PyMOL software (www.pymol.org). PRKACA in green, PRKAR1A in pink, K84 as yellow sticks, PRKAR1A-A100

regulatory subunits (R).[3] It has been found that the main catalytic subunit of PKA is PRKACA, and the main regulatory subunit is PKA regulatory subunit 1α (PRKAR1A). The position of PRKACA K84 in PKA holoenzyme (PDB:3FHI)[4] was depicted in Figure 1D. K84 was located at the interface between PRKACA and PRKAR1A, and it interacted with A100 and V102 of PRKAR1A through hydrogen bonds.

To clarify the role of PRKACA K84 Kcr, we produced a K84 glutamine mutant (K84Q) as a protein hypercrotonylation mimic and mutated K84 lysine to arginine (R) to mimic the decrotonylation of PRKACA K84. The levels of crotonylation of mutants were significantly lower than that of the WT (Fig. S5C), indicating that K84 is the main crotonylation site of PRKACA. Compared with WT and K84R, the binding ability of PRKACA K84Q to endogenous PRKAR1A decreased significantly in three independent transfections (Fig. 1E). PRKACA K84Q cells showed higher PKA activity than the WT, while K84R led to an opposite result (Fig. 1F; Fig. S5D). Consistent with PKA activity, K84Q increased the Ser133 phosphorylation level of cAMP response element binding protein (CREB) (Fig. S5E). PKA inhibitors H89 inhibited the activation of PKA in K84Q cells without forskolin, but R subunit cAMP binding competitive inhibitor (RP-cAMPS) had no significant effect (Fig. 1G; Fig. S5F). It is known that binding of two cAMP molecules to the R subunit can lead to conformation change of PKA holoenzyme and release of the C subunit which then phosphorylates the downstream target molecules. We speculated that the activation of PKA activity by K84Q may be structural, that is, K84 Kcr reduces the binding of regulatory subunits to catalytic subunits.

We found that the proliferation rates, colony growth, invasion and migration of CRC cells with K84Q overexpression were significantly higher than those of WT cells, while decreasing in K84R cells (Fig. 1H–K; Fig. S6A–D). RNA-seq data showed that 97 genes were upregulated and 162 were downregulated between PRKACA WT and K84Q (Fig. S7A). The enrichment of these genes nominated tumor-related pathways and functions (Fig. S7B, C) such as PI3K-Akt pathway which can be activated by focal adhesion kinase (FAK).[5] In addition, K84Q elevated the mRNA levels of cyclin E2 (CCNE2), a classic gene associated with tumor cell growth (Fig. S7D, E). The phosphorylation levels of FAK at Y397, Akt at S473, Paxillin (a focus adhesion marker) at Y118, and the protein level of CCNE2 increased in K84Q cells (Fig. 1L; Fig. S7F), which can be reversed by PKA inhibitor H89 (Fig. S7G). These results indicate that PRKACA K84Q enhances PKA activity and phosphorylates FAK and Akt, resulting in downstream CCNE2 expression and Paxillin phosphorylation in a PKA-dependent way.

The level of PRKACA Kcr in the tumor tissue was relatively higher than that in the matched peri-tumor tissue, while the levels of PRKACA did not show statistical significance (Fig. 1M; Fig. S8A, B). The levels of PRKACA Kcr did not show significant difference regarding TNM stage, gender, age, tumor size, tumor differentiation, lymph node metastasis, or distant metastasis (Fig. 1N and Table S3). These results suggest that PRKACA Kcr could be a diagnostic marker for CRC, but not associated with the clinicopathologic characteristics of CRC.

To date, the impact of protein Kcr on cancers remains poorly understood. To our knowledge, the present work is the first quantitative analysis of Kcr in human CRC which provides a rich data set of crotonylome. The study suggests that PRKACA K84 Kcr promotes the growth, invasion, and migration of CRC cells by activating the PKA-FAK-AKT signaling pathway (Fig. 1O), and may serve as a diagnostic marker for CRC. These findings expand the field of protein crotonylation regulation and provide a potential target for the treatment of CRC.

Author contributions

J.-Y.H. and J.-M.C. conceived and designed the study. J.-Y.H. and S.-L.H. collected the CRC tissue samples. J.-Y.H., L.-J.G., J.S. and L.Z. conducted the immunostaining, western blotting and IP experiments and analyzed the data. J.-Y.H. and D.-P.W. performed the enzyme activity experiments and drafted the molecular modeling of PRKACA. J.-Y.H. and L.-J.G. performed bioinformatic data analyses. J.-Y.H., T.S., and J.-Y.S. performed the cell experiments. J.-Y.H. drafted the manuscript. J.M.C. and D.-P.W. guided and reviewed the manuscript. All authors read and approved the final manuscript.

and V102 as green sticks, and hydrogen bond as yellow dash dot lines. **(E)** HA-tagged PRKAR1A was expressed in HCT116 cells in indicated combinations. Cell lysates were immunoprecipitated with anti-FLAG and analyzed by Western blotting with antibodies specific for FLAG and PRKAR1A. **(F)** HCT116 cells were treated with control or PRKACA siRNA for 36 h before transfected with PRKACA WT or K84Q or K84R. After 48 h of transfection, cells were treated with 10 μM forskolin for 2 h. PKA activities were measured in the presence or absence of forskolin. **(G)** Indicated plasmids-transfected HCT116 were cultured with DMSO, H89 (10 μM), or Rp-cAMPS (100 μM) for 4 h after 48 h of transfection, and PKA activities were assayed. **(H)** HCT116 cells were treated with control or PRKACA siRNA targeting 3′-untranslated regions for 36 h before cells were transfected with PRKACA WT or K84Q or K84R, and cell proliferation was determined by CCK8 assay. **(I)** Colony formation assay of HCT116, SW480, and SW620. Representative images of colony are shown. **(J)** Representative images of invaded cells in the Transwell assay. Scale bar: 100 μm. **(K)** Cell migration ability was assessed by wound healing assay in HCT116 cells. Representative images of wound area and percentage of wound gaps at the indicated time points after scratching are shown. Scale bar: 100 μm. **(L)** HCT116, SW480, and SW620 cells were treated with control or PRKACA siRNA for 36 h before cells were transfected with PRKACA WT or K84Q or K84R, and the expression of p-FAK, FAK, p-AKT, AKT, p-Paxillin, Paxillin, and cyclin E2 were assessed by Western blotting. β-Actin served as a loading control. **(M)** The protein levels of PRKACA and Kcr in CRC specimens and adjacent nontumorous tissues were detected by Western blotting ($n = 44$), and eight representative blots were presented. **(N)** Quantitative analysis of PRKACA among different progression stages. **(O)** Working model illustrates CBP and SIRT3 can mediate Kcr of PRKACA. Crotonylated PRKACA activates cyclin E2 gene expression and phosphorylation of Paxillin via PKA-FAK-AKT pathway to promote CRC development. Error bars represent mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, no significant difference.

Conflict of interests

The authors declare that they have no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.02.018.

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