Title：CYFIP1 and PKM2 synergistically regulate ROS and glycolysis in colorectal cancer through the AKT/mTOR/HIF-1α cascade

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Research plan

1.1 CYFIP1 is highly expressed in colorectal cancer

(1) Bioinformatics + clinical information + clinical tissue evidence show that CYFIP1 is highly expressed in colon cancer: Based on the GEPIA system (http://gepia.cancer-pku.cn/detail.php?gene=CYFIP1###), the expression level of CYFIP1 in colorectal cancer tissues was analyzed to be higher than that in benign colorectal tissues; Detection of the expression of CYFIP1 in human colorectal tissue specimens was conducted to further verify the cell line data. We collected 64 colorectal cancer patients and adjacent tissues for RT-qPCR and IHC verification; Analysis of the clinical pathological characteristics and survival curves of the collected colorectal cancer patients proved the relationship between the expression of CYFIP1 and tumor size, tumor stage, lymph node metastasis, and disease-free survival period.

(2) Determine the colorectal cancer cell lines to be used in subsequent experiments: We conducted Western blot analysis of CYFIP1 protein expression in common colorectal cancer cell lines HT29, SW480, SW620, HCT116, and normal colon cells NCM460.

1.2 CYFIP1 Affects Oxygen Metabolism and Glycolysis in Colorectal Cancer

(1) Whether CYFIP1 alteration affects oxygen metabolism in colorectal cancer: By infecting the CYFIP1 colon cancer cell line SW480 with a slow virus, and conducting subsequent sequencing analysis, we found that after knockdown of CYFIP1, there was a significant enrichment in the HIF pathway, and the oxygen metabolism function of the cells changed significantly; through Western blot, RT-qPCR, etc. verification of the infected SW480 and HT29 cells, it was confirmed that the transcription and translation levels of HIF-1α and related oxygen metabolism proteins were changed; further through experiments such as cell ROS and electron microscopy, it was confirmed that the damage to mitochondria and the generation of reactive oxygen species were affected; using Seahorse XF to measure the oxygen consumption rate of the cells; using kits to measure the ATP and pyruvate contents of colorectal cancer cells transfected with CYFIP1.

(2) Does the alteration of CYFIP1 affect the glycolysis of colorectal cancer and whether it directly binds to PKM2: When measuring the common metabolic substrates of oxygen

metabolism and sugar metabolism such as ATP, glucose, and lactate, we found significant changes in the products related to glycolysis. Based on this, after conducting metabolic group sequencing analysis, we discovered obvious abnormalities in metabolites such as pyruvate, and the glycolysis pathway was significantly enriched. Through Western blot and RT-qPCR, we confirmed that the expression of PKM2 decreased but its transcription was not affected. We verified the relationship between CYFIP1 and PKM2 using methods such as CH-IP, mass spectrometry, and immunofluorescence, and explored whether the interaction between the two affected the degradation of PKM2 and thereby influenced the glycolysis of colorectal cancer; Using PKM2-IN-1 (a PKM2 inhibitor) to inhibit the overexpressed CYFIP1-infected SW480 and HT29 cell lines, we measured the glycolytic metabolic products again and conducted reverse functional verification.

1.3 The specific mechanism by which CYFIP1 affects oxygen metabolism in colorectal cancer and the interaction between oxygen metabolism and glycolysis

(1) Determine the mechanism by which CYFIP1 affects oxygen metabolism in colorectal cancer through the AKT/MTOR/4EBP1/HIF-1 α pathway: Based on sequencing results and literature review, the AKT/MTOR/4EBP1/HIF-1 α pathway was the focus of the study; after overexpression and interference of CYFIP1 expression in colon cancer cells, Western blot was used to verify the protein of the entire AKT/MTOR/4EBP1/HIF-1 α pathway, verifying the completeness and consistency of the entire pathway; in the overexpressed colon cancer cell line, rapamycin (a MTOR inhibitor) was used to verify the changes in ROS, ATP, and related pathway proteins;

(2) Verify that CYFIP1 affects HIF-1 α through PKM2 nuclear translocation and influencing MTOR expression, thereby causing the intersection of glycolysis and oxygen metabolism: To verify the mechanism by which CYFIP1 causes simultaneous changes in oxygen metabolism and glycolysis after modification, immunofluorescence was used to observe the changes in the nuclear transfer of PKM2 in overexpressed and interfered SW480 and HT29 cell lines (the nuclear transfer of PKM2 will affect the transcription of HIF-1 α , thereby causing an interaction between glycolysis and oxygen metabolism); in the colon cancer cell line overexpressing CYFIP1, PKM2-IN-1 was used to verify the changes in AKT/MTOR/4EBP1/HIF-1 α pathway proteins,

ROS, ATP, and mitochondrial function, and rapamycin was used to observe the changes in PKM2 expression;

(3) Verify that the lactic acid produced by the change in PKM2 caused by CYFIP1 leads to the lactylation of HIF-1α and stabilizes HIF-1α, reducing its degradation: Overexpress CYFIP1 in HT29 and SW480 cells, and use the L-lactic acid kit to prove that the expression of L-lactic acid increased; in the colon cancer cell line with knocked-down CYFIP1, L-lactic acid was added, and the expression of HIF-1α protein was determined, proving that L-lactic acid can rescue the decrease in HIF-1α; WB was used to verify the expression levels of Pan-Kla and Pan-UB proteins, proving that L-lactic acid increased overall lactylation and simultaneously reduced overall ubiquitination; normal colon cancer cells were added with L-lactic acid, and the protein amount of HIF-1α was measured at different times, proving that L-lactic acid can change the half-life of HIF-1α; LC-MS/MS was used to reveal the lactylation sites of HIF-1α, molecular docking was used to simulate the binding conformation of HIF-1α and L-lactic acid, indicating that L-lactic acid binds at which sites; in summary, it is indicated that after the change of CYFIP1, PKM2 undergoes corresponding changes, and the lactylation of HIF-1α mediated by L-lactic acid plays a direct stabilizing role in HIF-1α expression.

1.4 CYFIP1 affects the immune microenvironment and chemotherapy resistance of colorectal cancer through metabolic reprogramming of oxygen metabolism and glycolysis.

(1) CYFIP1 affects the immune microenvironment of colorectal cancer through metabolic reprogramming: The measurement of lactic acid and HIF-1α lactylation indicated that lactic acidification occurred in colorectal cancer after CYFIP1 modification, and it was found from literature that the production of lactic acid would affect the immune microenvironment of the tumor; THP1 cells were co-cultured with CYFIP1-modified colorectal cancer cells and the following experiments were completed: WB verified the changes in MCT1 and MCT4, which verified the changes in the ability of colorectal cancer cells and THP1 cells to take up and excrete lactic acid; WB verified the changes in PD-L1 and PD-1 proteins; Flow cytometry verified the expression levels of CD80, CD86, CD163, and CD206 in THP1 cells, proving the changes in the proportion of M1 and M2 cells in immune cells after CYFIP1 modification. In the co-culture environment of CYFIP1-knocked-down colorectal cancer cell lines and THP1 cells,

L-lactic acid was added, and in the co-culture environment of overexpressed CYFIP1 colorectal cancer cell lines and THP1 cells, PKM2-IN-1 and rapamycin were added to repeat the above experiments again to complete the functional rescue experiments; Finally, the CSCs change was detected through tumor microsphere formation test;

(2) CYFIP1 affects the chemotherapy resistance of colorectal cancer through metabolic reprogramming: SW480 cells were continuously stimulated with cisplantin chemotherapy for 6 months to obtain stable drug-resistant SW480 cells, namely SW480/DDP (hereinafter referred to as DDP); CCK8 was used to detect the cell viability of DDP and SW480 cells at different drug concentrations, and the IC50 values were compared; Stable knockdown of CYFIP1 and stable high expression of CYFIP1 in SW480, HT29 cells, and stable knockdown of CYFIP1 in DDP cells were constructed by cell transfection technology, and their cell viability at different drug concentrations was detected by CCK8 technology; The tumor stem cell situation was verified through tumor microsphere formation test; The lymphocyte separation technique was used to simulate the in vivo microenvironment, and the changes were detected subsequently, while comparing the responses of immune microenvironment after CYFIP1 modification in resistant cells and normal cells; The immunohistochemical staining technique was used to verify the CYFIP1, KI-67, AKT/MTOR/HIF-1α pathways and PKM2-related proteins in the tumor.

1.5 CYFIP1 affects the proliferation and apoptosis of colorectal cancer through metaboli c reprogramming of oxygen metabolism and glycolysis.

(1) CYFIP1 affects the proliferation of colorectal cancer through metabolic reprogrammin g: After knockdown and overexpression of SW480 and HT29 cells, CCK8, TRANSWELL, sc ratch, clone and other experiments were conducted to verify the effect of CYFIP1 modi fication on the functions of colorectal cancer cell lines; in the overexpressed cell lines of SW480 and HT29, using PKM2-IN-1 and rapamycin respectively, the differences in CC K8 and TRANSWELL disappeared, and after 8 hours of hypoxia in the knockdown cell li nes of SW480 and HT29, the differences disappeared again, and the differences appear ed again after reoxygenation; the in vitro animal experiments were divided into four gr oups: knockdown, overexpression, overexpression + PKM2-IN-1, overexpression + rapamy cin, control group, control + PKM2-IN-1, control + rapamycin; after knockdown and over expression, there were differences in subcutaneous tumor formation, and after adding PKM2-IN-1 and rapamycin injections, the differences disappeared; for the tumor tissues of animals, HE staining, CYFIP1, Ki67, PKM2, HIF-1α and other protein immunohistoch emical staining were performed, and CYFIP1 and PKM2 were double-stained by immuno fluorescence.

(2) CYFIP1 affects the apoptosis of colorectal cancer through metabolic reprogramming: The results of bioinformatics prediction of the correlation between CYFIP1 and apoptosis proteins were obtained; after knockdown of CYFIP1 in SW480 and HT29 cells, flow cytometry was used to verify whether apoptosis increased; the results of related apoptosis proteins such as BCL2, caspase3, c-caspase3 were obtained, and PKM2-IN-1 and rapamycin were used in the overexpressed cell lines of SW480 and HT29 again for verification; the expression of apoptosis proteins was verified again in the animal tumor tissues using Western blot and immunohistochemistry methods.

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Ethics approval and consent to participate

The study was ratified by the Ethics Committee of the Second Affiliated Hospital of Anhui Medical University and conducted in compliance with the Declaration of Helsinki, and the ethics number was (YX2023-183). Written informed consent form was obtained from each eligible patient. The animal study protocol was ratified by the Animal Ethics Committee of the institute, and all procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, Maryland, USA).