

The Expression Characteristics of the RBFOX1 Gene in Colorectal Cancer

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Abstract

Introduction: Copy number variation is a significant characteristic of colorectal cancer progression. RBFOX1 (A2BP1) is the gene with the highest frequency of copy number loss in colorectal cancer, but current research related to it and colorectal cancer is relatively scarce.

Methods: Data from TCGA and other sources were used to analyze the copy number variation and mRNA expression levels of RBFOX1, as well as their correlation with clinical pathological data. Immunohistochemistry and immunofluorescence experiments were used to analyze the expression of RBFOX1 protein in colorectal cancer cells and tissues.

Results: RBFOX1 has a high frequency (22.4%) of copy number loss and diverse copy number variations in colorectal cancer tissues. High-level RBFOX1 deletion is prone to occur in the right-sided colon and tissues with high microsatellite instability. The copy number variation of RBFOX1 and mRNA expression are not correlated. In tumor tissues, RBFOX1 mRNA shows a characteristic of reduced expression, which is significantly related to BRAF mutation ($P=4.7e-05$, $P=0.03$). Low expression of RBFOX1 is prone to occur in the right-sided colon and tissues with high microsatellite instability. The protein encoded by RBFOX1 is expressed in normal intestinal tissues, but shows a characteristic of absence in some colorectal cancer tissues.

Conclusion: In the right-sided colon and tissues with high microsatellite instability, RBFOX1 shows copy number loss and low mRNA expression. This characteristic is closely related to BRAF gene mutation, and the protein of RBFOX1 is absent in some colorectal cancer tissues.

Keywords

colorectal cancer, copy number variation, copy number loss, RBFOX1

Introduction

Colorectal cancer is a prevalent malignant tumor that poses a significant threat to human health.¹ It is a heterogeneous disease, and a single fixed treatment approach cannot benefit all patients. Therefore, the current research focus is on classifying different tumor patients into subtypes based on characteristics such as gene mutations, chromosomal structural changes, and epigenetic factors, in order to provide more targeted and precise treatments for patients of different subtypes.² Among all molecular classifications, copy number variation (CNV) is an extremely important indicator. For instance, in consensus molecular subtypes, CMS2 and CMS4 exhibit significant copy number alterations.³ Additionally, through the use of tumor organoids, scientists have discovered that individual and multiple mutations of genes such as APC and TP53 do

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not seem to cause distant metastasis of tumors, whereas the combination of these gene mutations with chromosomal instability can lead to significant metastasis. These findings highlight the crucial role of chromosomal instability, particularly CNV, in the progression of colorectal cancer.^{4,5}

In our previous work, we utilized data from TCGA and other sources to analyze the characteristics of the most prominent copy number amplification region, 20q, in colorectal cancer. We identified several oncogenes located within this region.^{6,7} The study of 20q and its relationship with colorectal cancer has gained increasing attention from researchers, and some studies have found that the presence of copy number amplification in 20q can divide colorectal cancer into two distinct subtypes.⁸ However, it is surprising that large data visualization websites for colorectal cancer, such as Cbioportal⁹ and COSMIC,¹⁰ show that the frequency of deep deletion of copy number in the 16p13.3 region ranks first, significantly higher than 20q. Nevertheless, there is relatively limited comprehensive research on the relationship between the 16p13.3 region, the RBFOX1 gene located within it, and colorectal cancer. Therefore, in this article, we will utilize large-scale data and experimental analysis to investigate the CNV characteristics, copy number alterations, mRNA expression levels, and their correlation with clinical pathological data of the RBFOX1 gene in colorectal cancer. We will also employ experimental methods such as immunohistochemistry and immunofluorescence to analyze the expression of RBFOX1 protein in colorectal cancer cells and tissues. These studies will provide preliminary theoretical support for understanding the relationship between RBFOX1 and colorectal cancer.

Methods

Download of Colorectal Cancer Data and Copy Number Evaluation of RBFOX1

Two colorectal cancer datasets, namely Colorectal Adenocarcinoma (TCGA, Firehose Legacy) and Colon Cancer (CPTAC-2 Prospective, Cell 2019), were downloaded from The cBioPortal for Cancer Genomics official website.⁹ These two large datasets include copy number data, mRNA expression data, and clinical information of colorectal cancer samples. Putative copy-number calls on colorectal cancer datasets were determined using GISTIC 2.0. The values assigned are as follows: -2 = homozygous deletion; -1 = hemizygous deletion; 0 = neutral / no change; 1 = gain; 2 = high level amplification.¹¹

Clinical Sample Collection

Colorectal cancer and adjacent tissue samples were obtained from surgical specimens at the Department of Colorectal and Anal Surgery, Zhongnan Hospital of Wuhan University, from April 2024 to October 2024. These specimens were immediately fixed in 4% formaldehyde after being excised. Paraffin sections of normal colorectal tissue, human brain tissue, and colorectal cancer tissue microarrays were sourced from the

Pathology Department of Zhongnan Hospital of Wuhan University. The use of all these samples has been approved by the ethics committee of Wuhan University (Approval No. 2024042 K), and informed consent was obtained from all patients prior to surgery.

Immunohistochemistry

The selected paraffin blocks were sliced into sections with a thickness of approximately 4µm and baked in a constant temperature oven at 80 °C for half an hour. Then, the sections were dewaxed in water; antigen retrieval was performed in a high-pressure cooker with citrate repair solution (diluted 50X) for 1.5 min, followed by rinsing with running water until room temperature. The sections were outlined with an immunohistochemistry pen, rinsed with PBS, and then incubated at room temperature for 10 min with hydrogen peroxide solution (DAKO). The liquid was poured off, and the sections were rinsed with PBS three times. Blocking solution with sheep serum (Beijing Zhongshan) was added dropwise, and the sections were incubated for half an hour. The liquid was poured off, and the sections were rinsed with PBS three times. A2BP1 Polyclonal antibody (Proteintech, USA, 22647-1-AP) was added dropwise and incubated at 37 °C for 1 h. The liquid was poured off, and the sections were rinsed with PBS three times. HRP-labeled secondary antibody (DAKO, ready-to-use) was added dropwise and incubated at room temperature for 30 min. The liquid was poured off, and the sections were rinsed with PBS three times. DAB chromogenic reagent A and B (DAKO) were added for color development for 3 min, and the color development was observed under a microscope. The color development was terminated by rinsing with PBS, followed by counterstaining with Mayer's hematoxylin for 5 min, rinsing with water, and bluing. The sections were dehydrated with ethanol, air-dried, and then sealed with coverslips.

Gene Mutations and the Expression Level of RBFOX1

The correlation analysis of common colorectal cancer mutation genes, including TP53, APC, KRAS, SMAD4, BRAF, PI3KA, etc, and the expression level of RBFOX1 was conducted using the Gene Mutation module of TIMER2.0.¹² The Gene Mutation module can analyze the log2 fold changes of the differential expression of RBFOX1 for each cancer type, according to the writing characteristics of Nature magazine.

Microsatellite Instability Evaluation

The microsatellite instability evaluation of colorectal cancer samples from TCGA and CPTAC was comprehensively predicted through MSIsensor¹³ and PreMSIm.¹⁴ For the results predicted by MSIsensor, tissues with an MSI score <10 are defined as microsatellite stable (MSS), and those with an MSI score ≥10 are defined as microsatellite unstable (MSI-H). In the results predicted by PreMSIm, 1 represents MSI-H, while 0 represents MSS colorectal cancer tissue.

Cell Culture

Colorectal cancer cells HT-29 (ATCC accession number: HTB-38) and SW480 (ATCC accession number: CCL-228) were cultured with L15 culture medium (KeyGEN BioTECH, Nanjing, China) + 10% fetal bovine serum (FBS) (Biological Industries, Israel), colorectal cancer cells HCT116 were cultured with McCoy's 5A culture medium (KeyGEN BioTECH, Nanjing, China or Gibco, USA) + 10% FBS. All cells were cultured in a cell incubator at 37°C with 5% CO₂. All cells were donated by the Department of Gastroenterology, Zhongnan Hospital of Wuhan University.

Immunofluorescence

Small round slides were soaked in 70% ethanol for more than 5 min, dried under a sterile ultra-clean table, and washed three times with disposable PBS. The washed slides were placed in a 24-well plate, and then the experimental cells were inoculated. After the cells were placed in the cell incubator overnight, the following experiments were performed when the cell fusion degree was 50% to 80%. The culture medium was aspirated clean, and the fixative polyformaldehyde was added to the 24-well plate. The cells were fixed overnight. The next day, the fixative was discarded, and the cells were washed 2–3 times with washing solution on a shaker, each time for 5 min. Then, the cells were sealed with sealing solution on a shaker for 1 h. After removing the sealing solution, the diluted A2BP1 Polyclonal antibody (Proteintech, USA, 22647-1-AP) was applied at 4°C overnight. After overnight incubation, the primary antibody was washed off with washing solution, and after washing, the washing solution was discarded and the fluorescently labeled secondary antibody was added and incubated for more than 1 h. The cells were washed three times with washing solution on a shaker (5 min each time), and light avoidance operation was noted. DAPI was dropped onto the slide to stain the cell nucleus for about 15 min. After washing three times, the cover slide in the 24-well plate was taken out, air-dried, and placed on the slide. The cells were sealed with anti-fluorescence quenching agent, and the cell fluorescence could be observed and recorded under a fluorescence microscope.

Statistical Analysis

The chi-square test and two-tailed unpaired Student's t-test were carried out using GraphPad Prism 9.5 software. The overall survival (OS) and disease-free survival (DFS) curves were generated using the log-rank test in accordance with the Kaplan-Meier method, utilizing R 4.2.1 software. A simple linear regression model was employed to analyze the correlation between the expression of mRNA and the CNV of RBFOX1. All data were replicated at least three times independently. The threshold for statistical significance was set at $P < 0.05$.

Results

RBFOX1 Exhibits High Frequency Copy Number Deletions in Colorectal Cancer Tissues

Online visualization images from Cbioportal show that RBFOX1 has the highest frequency of copy number deletions in colorectal cancer (Figure 1A). We then downloaded TCGA data to further analyze the copy number characteristics of RBFOX1. The results show that deep copy number deletions are not the only feature of this gene. In 616 cases of colorectal cancer tissues, deep copy number deletions account for 22.40% (138/616), low-level copy number deletions account for 14.61% (90/616), and 46.10% (284/616) did not undergo copy number changes. Additionally, some colorectal cancer tissues also exhibited copy number amplifications, with low-level copy number amplifications accounting for 16.23% (100/616), and high-level copy number amplifications accounting for 0.65% (4/616) (Figure 1B). To validate this trend, we used the Colon Cancer (CPTAC-2 Prospective, Cell 2019) dataset for similar analysis. The results showed that deep copy number deletions accounted for 23.81% (25/105), low-level copy number deletions accounted for 14.29% (15/105), 58.10% (61/105) did not undergo copy number changes, low-level copy number amplifications accounted for 2.86% (3/105), and high-level copy number amplifications accounted for 0.95% (1/105) (Figure 1C). These results indicate that RBFOX1 exhibits high frequency copy number deletions in colorectal cancer, but also presents diverse characteristics.

RBFOX1 Copy Number Variation is Correlated with Clinical Pathology

Given the different copy number characteristics of RBFOX1, we divided RBFOX1 into four groups according to the changes in copy number (Group A represent high-level copy number deletions group, Group B represent low-level deletion group, Group C represent normal group, Group D represent copy number amplification group), and statistically analyzed the correlation between these four groups and 13 clinical information. The results from Table SI and Table 1 show that tumor location and microsatellite instability are the two most statistically significant clinical pathological indicators. Specifically, compared to the normal group, low-level deletion group, and copy number amplification group, high-level copy number deletions are more likely to occur in the right-sided colon. High microsatellite stability is more likely to occur in the group with high-level RBFOX1 deletions compared to the normal group, the group with mild copy number deletions, and the group with copy number amplifications. Both copy number amplification and deletion seem to lead to tumor progression. Compared to the normal copy number group, low-level copy number deletions are more likely to occur in later stages, with lymph node metastasis, low microsatellite instability, and invasion of the peritoneal tissue. Compared to the normal copy number group, copy number amplifications are more likely to occur in later stages, with lymph node metastasis, low microsatellite instability, and tissues where both blood vessels and lymph nodes are invaded by the tumor. In addition, we found some other potentially important

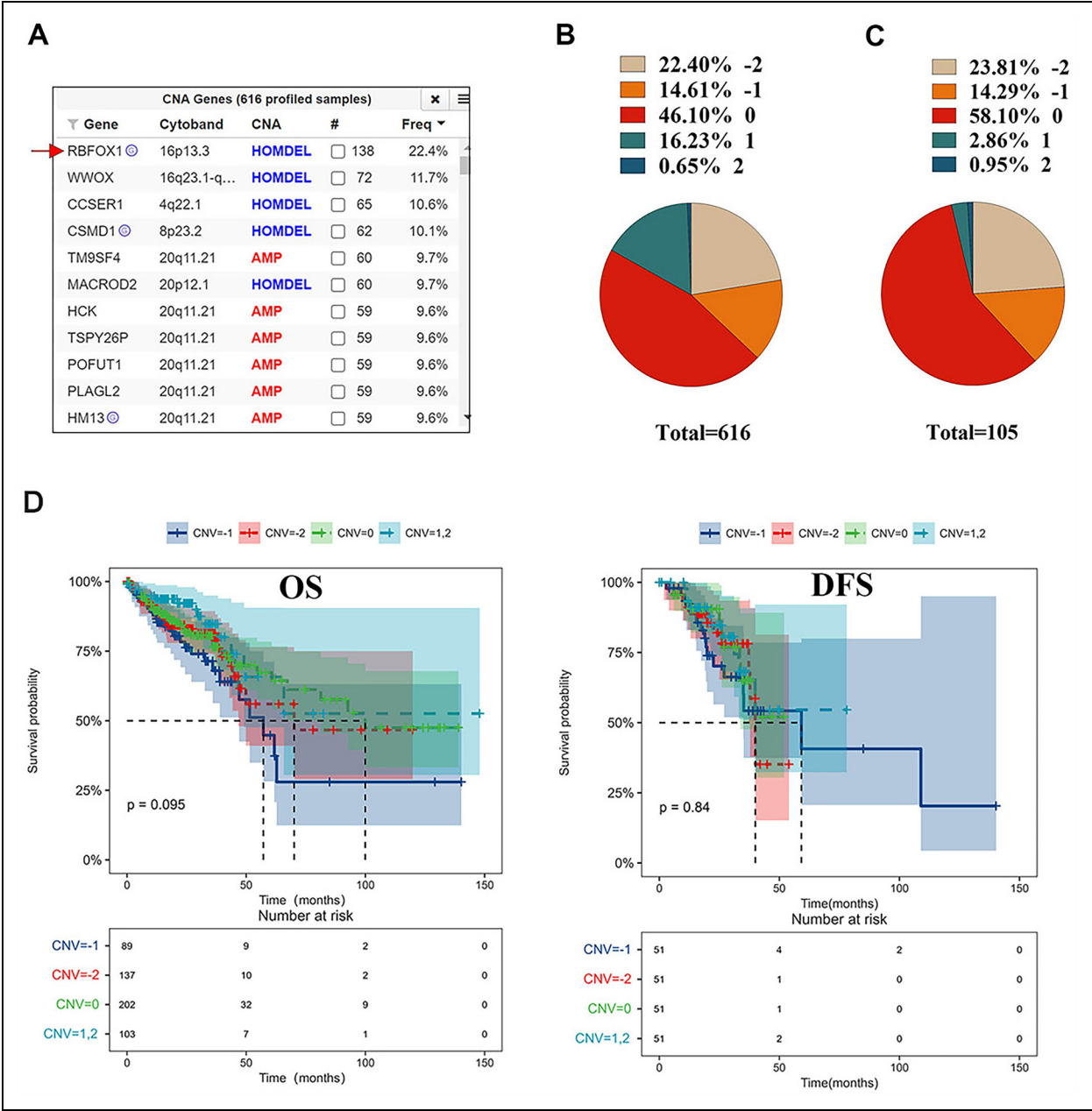


Figure 1. Copy Number Variation of RBFOX1 and Its Correlation with DFS and OS. A. Online Data from Cbioportal shows that RBFOX1 has the Highest Frequency of Copy Number Deletions in Colorectal Cancer. B And C. Analysis of RBFOX1 Copy Number Situation in Colorectal Adenocarcinoma (TCGA, Firehose Legacy) and Colon Cancer (CPTAC-2 Prospective, Cell 2019) Data. D. KM Survival Curve of Progression-Free Survival and Overall Survival Among the Four Groups.

statistical differences. In tissues where the lymph nodes are invaded by the tumor, different levels of copy number changes are more easily observed. We performed survival analysis on the progression-free survival and overall survival between the four groups, and tested them with the Log-rank test. The results show that there is no statistical significance between the four groups in DFS ($P=0.84$) and OS ($P=0.095$). Survival analysis was also performed between each of the two groups (results not shown), and no correlation was found between RBFOX1 CNV and the prognosis of colorectal cancer (Figure 1D).

The Copy Number Variation of RBFOX1 and mRNA Exhibit Independent Characteristics

Based on the characteristics of RBFOX1 copy number, we further analyzed the CNV-mRNA synergy of the RBFOX1 gene. Among 376 colorectal cancer tissues that included both mRNA and CNV values, the results showed that the two were not related (Figure 2A). Surprisingly, we found that compared with tissues without copy number changes, tissues with copy number loss, especially deep copy number loss, had increased

Table 1. Perform Statistical Analysis of the P Values for Inter-Group Comparisons Based on the Grouping Results Shown in Table SI, A Represent High-Level Copy Number Deletions Group, B Represent Low-Level Deletion Group, C Represent Normal Group, D Represent Copy Number Amplification Group.

	A versus B	A versus C	A versus D	B versus C	B versus D	C versus D
C or R						
age	0.6578	0.2586	0.9256	0.3514	0.7298	0.166
sex	0.2248	0.8511	0.1686	0.1304	0.0177	0.1649
site	0.0003	0.0039	<0.0001	0.1081	0.853	0.0592
N0 versus N1 + N2	0.196	0.3365	0.0646	0.0225	0.6555	0.0029
T2 + T1 versus T3 + T4	0.6607	0.0805	0.2815	0.3102	0.3006	0.6844
M	0.2635	0.9905	0.2689	0.1997	0.9632	0.2001
STAGE I+II versus III+IV	0.2416	0.1947	0.0636	0.0159	0.5687	0.0011
Vascular invasion	0.8485	0.0582	0.2011	0.0663	0.3448	0.0012
Lymphovascular invasion	0.875	0.0317	0.142	0.0466	0.2487	0.0003
Perineural Invasion	0.0304	0.8633	0.2827	0.018	0.2736	0.28
Weight	0.506	0.0535	0.9184	0.1879	0.4565	0.0345
Patient Height	0.6069	0.6653	0.668	0.91	0.3533	0.3632
MSI-S versus MSI-H	<0.0001	0.0007	<0.0001	0.0086	0.5568	0.0014

mRNA expression ($P < 0.05$), and copy number amplification also had the characteristic of increased mRNA expression (Figure 2B). This seems to be a contradictory conclusion. To better understand the CNV-mRNA characteristics of the RBFOX1 gene, we separately analyzed the correlation of 4 groups of CNV-mRNA, and the results showed that CNV and mRNA were not related (Figure 2C-F). These results indicate that the copy number changes of RBFOX1 in colorectal cancer cannot lead to corresponding changes in mRNA dosage. We then analyzed the expression level of RBFOX1 in tumor and normal tissues, and the results showed that the mRNA of RBFOX1 was down-regulated in tumor tissues (Figure 2G). According to the expression level of mRNA, colorectal cancer samples were divided into high mRNA expression group and low mRNA expression group, and the correlation between these two groups and 13 clinical samples was statistically analyzed. The results showed that low expression of RBFOX1 tended to occur in the right-sided colon and tissues with high microsatellite instability (Table 2). In addition, we also analyzed the correlation between the expression level of RBFOX1 and common mutation genes in colorectal cancer. The results showed that the expression of RBFOX1 was reduced in colorectal cancer tissues with BRAF mutations, but it was not related to TP53, APC, KRAS and SMAD4 (Figure 2H).

The Protein RBFOX1 is not Expressed in Some Colorectal Cancers

The immunofluorescence of the protein database¹⁵ shows that RBFOX1 presents a certain degree of difference in cancer cells of different tissues. In human osteosarcoma cells U-2 OS, RBFOX1 has a uniform nuclear distribution characteristic. In human glioma cells U251, the protein has a nuclear distribution characteristic, but the nuclear intensity is significantly stronger than the cytoplasm. In human rhabdomyosarcoma cells RH-30, RBFOX1 protein is mainly distributed in the cell nucleus and

Golgi apparatus (Figure 3A). Next, we analyzed the characteristics of RBFOX1 protein in colorectal cancer cells. The COSMIC Tumor Cell Project reveals that copy number losses are present in multiple colorectal cancer cell lines, including HCT-116 and HT-29 (Figure 3B). Immunofluorescence shows that RBFOX1 protein is mainly distributed in the nucleus in well-differentiated tumors such as HT-29 and SW480, while in poorly differentiated tumors such as HCT116, the protein is mainly distributed in the nucleus, and a small amount of it is distributed in the cytoplasm (Figure 3C). The immunohistochemistry results of the protein database¹⁵ show that the protein is mainly distributed in the glandular cells of the normal intestine and shows a moderate intensity of cytoplasmic distribution characteristics, but the expression of this protein was not detected in 11 cases of colon cancer tissues (0/21, 0%) (Figure 4A). To confirm this conclusion, we collected a normal brain sample as a positive control from the clinic, analyzed the intestinal tissue of a patient who underwent total colectomy due to chronic transmission constipation, three right colon cancers and adjacent tissues, and performed RBFOX1 immunohistochemical staining on the colorectal cancer chip. The results showed that RBFOX1 in brain tissue is mainly located in neurons and nuclear staining, which indicates that the antibody has specificity (Figure 4C). The constipation intestinal tissue and adjacent tissues showed the same characteristics as the protein database (Figure 4D/E), but we found that the cancer tissues all had protein staining (Figure 4E, Table SII). In two high-throughput pathological tissue chips (consisting of 69 cases of colorectal cancer tissues), three cases of cancer tissues lacked expression, and other cancer tissues all had expression (Figure 4B).

Discussion

RBFOX1 encodes an RNA-binding protein that regulates the specific splicing of many crucial neuronal transcription factors.

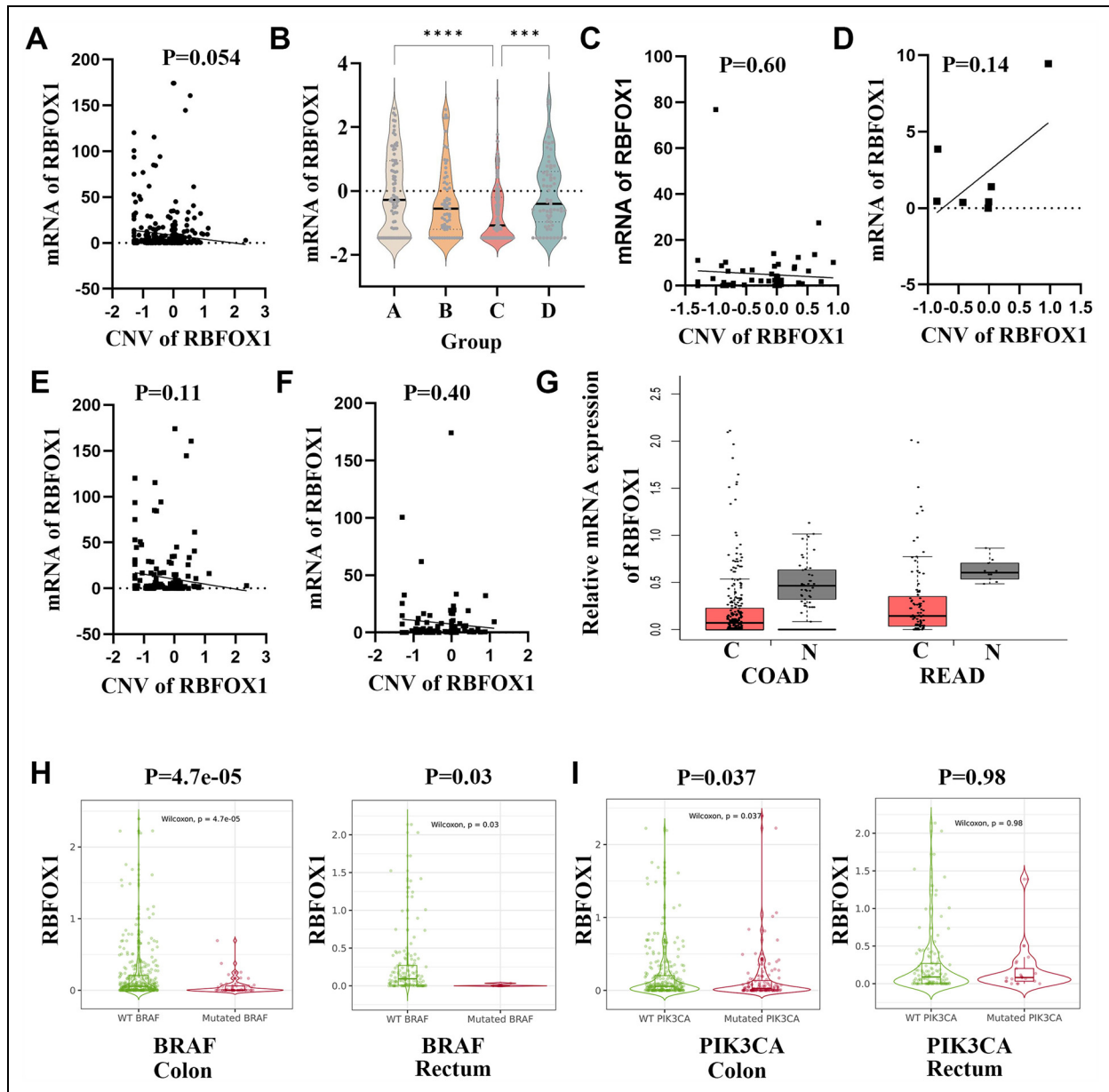


Figure 2. Characteristics of RBFOX1 mRNA in Colorectal Cancer. A. Correlation Analysis of Total RBFOX1 Copy Number Variation and RBFOX1 mRNA Expression. B. Differences in RBFOX1 mRNA Expression Among the Four Groups, with Significant Differences Between Groups A And C (**** Represent $P < 0.0001$), and Significant Differences Between Groups C And D (*** Denotes $P < 0.001$). C-F. Correlation Analysis of Copy Number Variation and Corresponding mRNA Expression in Each Group Among the Four Groups, C: (Group A–High–Level Copy Number Deletions Group), D(Group B– Represent Low-Level Deletion Group), E(Group C– Normal Group), F(Group D –Copy Number Amplification Group). G. Relative mRNA Expression of RBFOX1 in Colon Adenocarcinoma and Rectal Adenocarcinoma, Indicating that RBFOX1 mRNA Expression in Colorectal Cancer is Lower than in Normal Tissues. H. Correlation between BRAF Gene Mutation and RBFOX1 mRNA in Colon Cancer and Rectal Cancer Tissues. I. Correlation Between PIK3CA Gene Mutation and RBFOX1 mRNA in Colon Cancer and Rectal Cancer Tissues.

Variations in its copy number can lead to severe diseases, including mental retardation, epilepsy, schizophrenia, and autism spectrum disorders.^{16–19} It has been found that RBFOX1 interacts with SCA2, a key gene causing spinocerebellar ataxia type 2,²⁰ and recent findings suggest that RBFOX1 may be a new locus involved in the pathogenesis of Alzheimer's disease.²¹ Additionally, some studies indicate that this protein may be knocked down in gliomas and other tumors.^{22–24}

In colorectal cancer, the RBFOX1 gene exhibits a high frequency of copy number deletions. Interestingly, the variation in the copy number of this gene in colorectal cancer tissues is diverse, with some tissues showing low levels of copy number deletions and amplifications. This feature is distinctly different from the copy number changes of other chromosomes. For instance, in colorectal cancer tissues, genes on 8q and 20q show significant copy number amplifications, which are

Table 2. Analyze the Correlation Between the mRNA Expression and 13 Clinical Information in the Colorectal Adenocarcinoma (TCGA, Firehose Legacy) Dataset.

	High	Low	P value
<50	19	38	0.1889
≥50	136	183	
male	87	120	0.7254
female	68	101	
Left hemicolon	92	99	0.017
Right hemicolon	51	94	
T12	25	42	0.4782
T34	129	178	
N0	81	125	0.3916
N1 + N2	73	94	
I, II	74	118	0.5288
III, IV	69	96	
NO vascular_invasion	105	144	0.9922
YES vascular_invasion	32	44	
NO Lymphovascular_invasion	99	129	0.2935
YES Lymphovascular_invasion	38	64	
NO perineural	65	101	0.225
YES perineural	28	30	
MSS	151	171	<0.0001
MSH	4	47	

singular and almost devoid of deletion mutations.^{25–27} Furthermore, when analyzing the correlation between RBFOX1 copy number changes and clinical information, we found that both copy number amplifications and deletions seem to lead to tumor progression. These findings suggest that although RBFOX1 exhibits significant copy number deletions in colorectal cancer, it may not be a typical tumor suppressor gene (TSG). Multiple studies have shown that RBFOX1 on chr-16 p region is often affected by heterozygous and homozygous deletions, but its homozygous deletion frequency is much lower than that of classical TSGs, and no somatic mutations have been observed in the RBFOX1 coding region, indicating that it is not a classical TSG.^{28,29}

In the analysis of the correlation between copy number and clinical pathological data, we made an important finding that the high-frequency copy number deletions of RBFOX1 mainly occur in the right-sided colon and in tissues with microsatellite instability. This suggests that the CNV of RBFOX1 may not be an isolated random event. In tumor tissues, changes in DNA copy number often accompany changes in mRNA and protein,³⁰ but we found that the copy number values of RBFOX1 do not correlate with mRNA expression levels. Moreover, regardless of copy number deletions or amplifications, the expression level of RBFOX1 is elevated, suggesting that the expression level of RBFOX1 in the progression of colorectal cancer does not seem to be regulated by copy number changes. This is distinctly different from our previous research on 20q. Genes on 20q in the progression of colorectal cancer follow a highly correlated CNV-mRNA-protein change pattern.^{6,7} Interestingly, we found that compared to normal tissues, the expression level of RBFOX1 is significantly reduced in

colorectal cancer tissues, and low expression of RBFOX1 mainly occurs in the right-sided colon and in tissues with high microsatellite instability.³¹

Importantly, we found that in colorectal cancer tissues, the expression level of RBFOX1 is significantly correlated with BRAF mutations, but not with TP53, APC, KRAS, and SMAD4. Combined with the analysis in the article, deep copy number deletions mainly occur in the right-sided colon and in tissues with high microsatellite instability. In subgroup analysis, there is a certain degree of significance in the CNV differences of RBFOX1 in females, and BRAF gene mutations are mainly related to females, usually on the right side of the colon and in the late stage, and are related to high microsatellite instability. These findings indicate that in colorectal cancer, the RBFOX1 gene is significantly correlated with BRAF mutations in colorectal cancer. Some studies have shown that RBFOX1 may inhibit the occurrence of gliomas by regulating many genes, one of which is that RBFOX1 partially inhibits the occurrence of gliomas by affecting the selective splicing of a recognized tumor suppressor factor TPM1.²³ Therefore, we speculate that the selective splicing of RBFOX1 may be involved in the mutation of the BRAF gene, resulting in such a high degree of clinical pathological characteristic correlation between the two.

RBFOX1 is mainly expressed in neurons, heart, and skeletal muscle.³² RBFOX1 not only acts as a splicing regulator in the nucleus but is also selectively spliced to produce a cytoplasmic form. Hu and colleagues' IHC results show that compared to normal tissues, RBFOX1 is expressed at lower levels in human gliomas.²³ Neel and colleagues' immunohistochemistry results show that RBFOX1 is expressed at low levels in the intestine, and the expression of this protein is reduced in colorectal cancer.²² Our database and IHC studies show that RBFOX1 protein is only expressed in some colorectal cancer tissues, which may be related to the patient's BRAF gene mutation.

In this article, we have only preliminarily explored the correlation between the RBFOX1 gene and colorectal cancer. Although compared to other oncogenes or TSGs, RBFOX1 does not seem to be typical, and some studies show that in tumors, the RBFOX1 region is prone to copy number deletions and does not have a certain correlation with tumor suppression. However, its changes mainly occur in the right-sided colon and in tissues with high microsatellite instability. This feature not only reflects the characteristics of BRAF mutations in colorectal cancer tissues but is also significantly correlated with this mutation. These findings suggest that RBFOX1 may be related to colorectal cancer. The gene database Genecard shows that RBFOX1 is a colorectal cancer-related "elite" gene and ranks 66th.

It is worth noting that this paper also has some shortcomings and limitations. Our validation was conducted solely on clinical specimens from a single-center study with a limited sample size, necessitating further verification through multi-center studies with larger sample cohorts in subsequent research phases. Current investigations into RBFOX1 and colorectal carcinoma remain relatively limited. Discrepancies observed in immunohistochemical analyses compared with previous

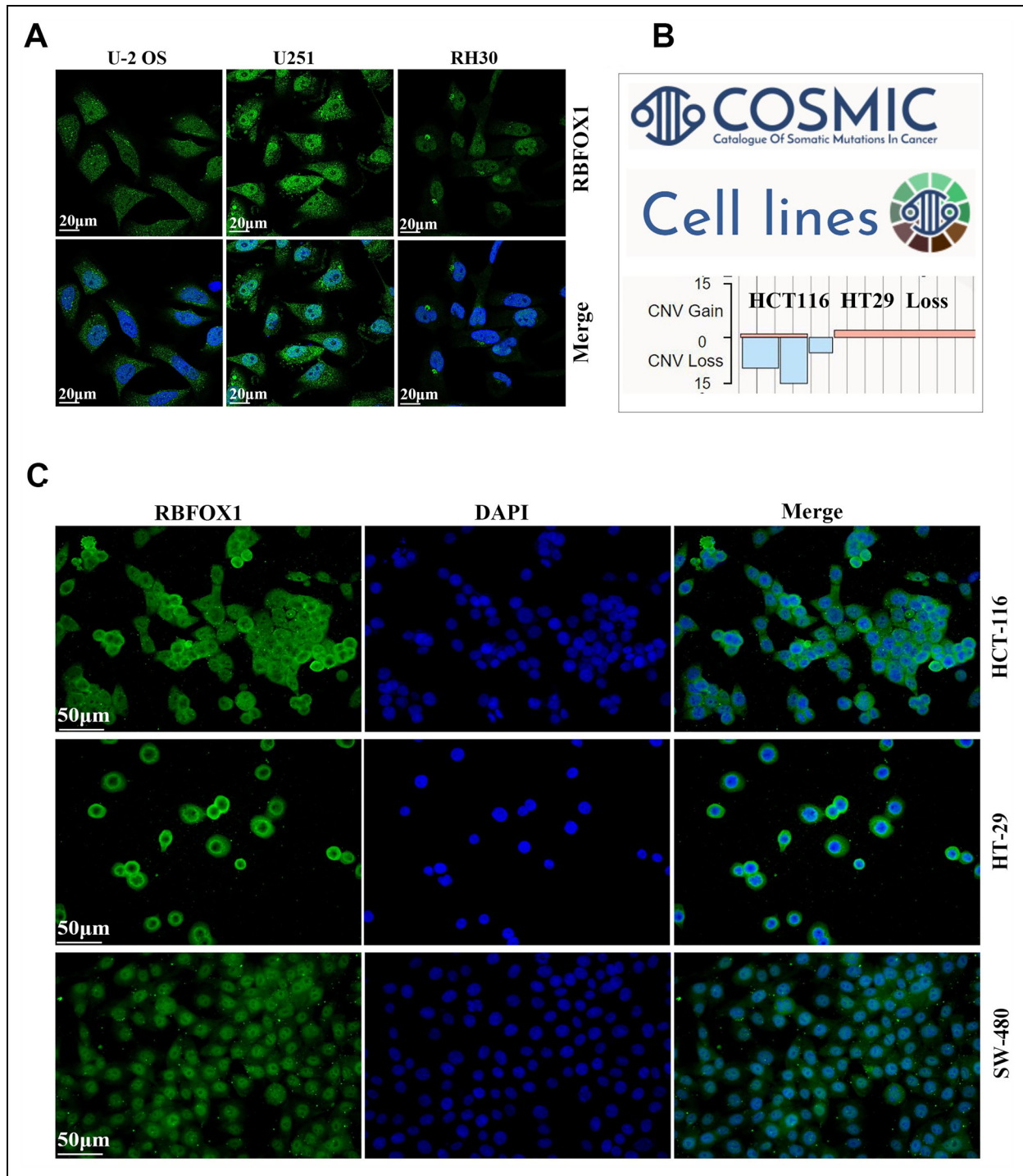


Figure 3. Immunofluorescence Analysis of RBFOX1 Protein in Tumor Cells. A. Immunofluorescence from the Human Protein Atlas Database shows that RBFOX1 has Different Nuclear-Cytoplasmic Distribution Characteristics in Cancer Cells of Three Different Tissues. (<https://www.proteinatlas.org/search/RBFOX1>) B. COSMIC Cancer Cell Engineering shows Copy Number Deletions in Multiple Colorectal Cancer Cells. C. Immunofluorescence shows that RBFOX1 Protein is Mainly Distributed in the Nucleus of Well-Differentiated Tumor Cells HT-29 and SW480, While in Poorly Differentiated HCT116, The Protein shows Nuclear-Cytoplasmic Distribution Characteristics.

studies, despite our utilization of brain tissue as positive controls, may potentially stem from antibody specificity issues and tumor heterogeneity in clinical sample acquisition. Independent validation of this antibody across different

research centers is therefore warranted. Although our findings suggest a potential association between RBFOX1 and colorectal carcinogenesis, it remains unclear whether this represents a primary causative event or a secondary epiphenomenon

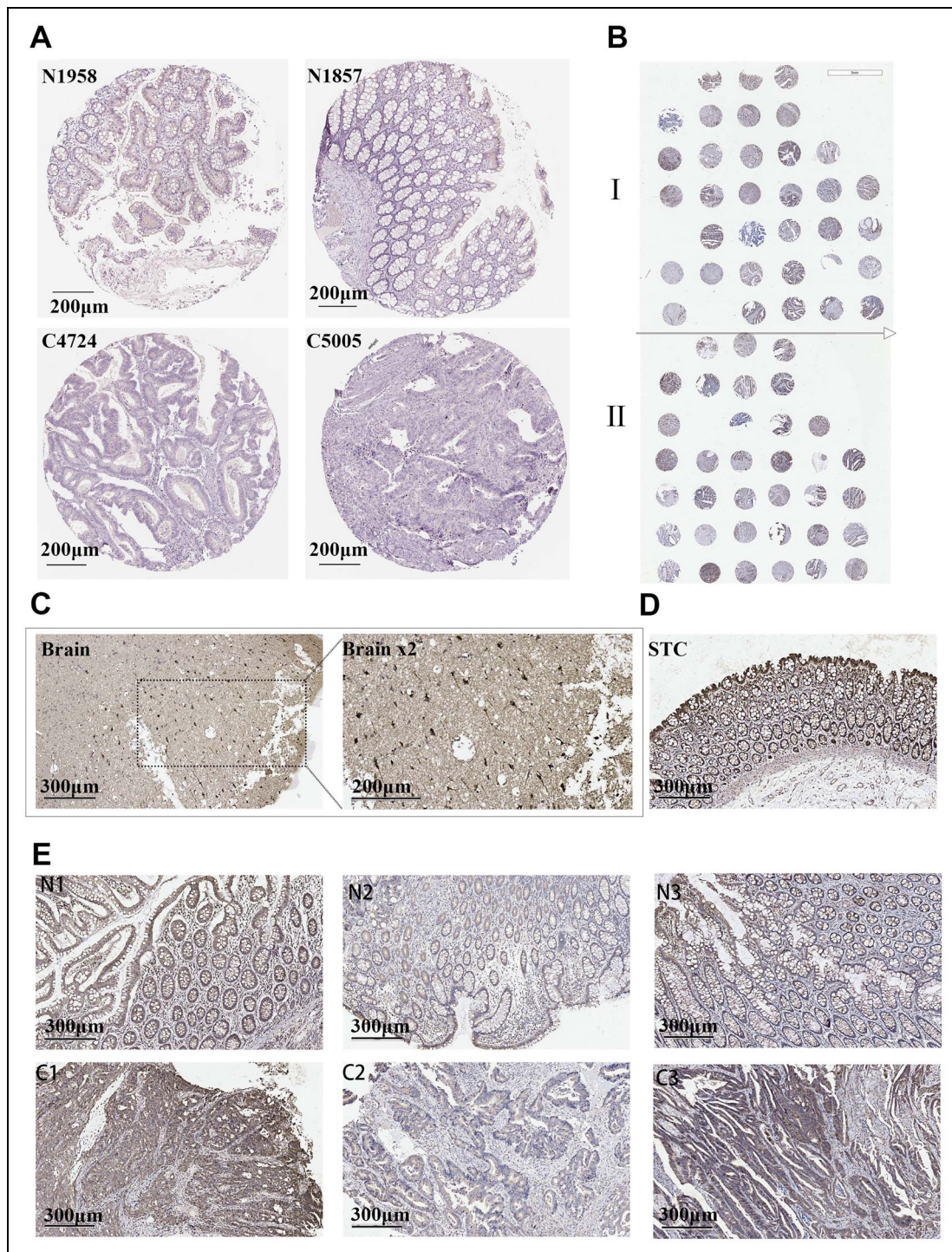


Figure 4. Immunohistochemical Analysis of RBFOX1 Expression Level in Normal and Cancerous Colorectal Tissues. A. Immunohistochemical Results from The Human Protein Atlas Database Show that the Protein is Mainly Distributed in the Glandular Cells of the Normal Intestine and shows Low Cytoplasmic Distribution Characteristics, But the Expression of this Protein is not Detected in Colorectal Cancer Tissues, Here are Two Representative Tissues. N1958 Representative an Immunohistochemical Results of RBFOX1 of 84 Years Old Female Patient in Normal Colon Tissue, 1958 is the Patient ID, N1857 Representative an Immunohistochemical Results of RBFOX1 of 47 Years Old Male Patient in Normal Colon Tissue, C4724 Representative an Immunohistochemical Results of RBFOX1 of 70 Years Old Female Patient in Adenocarcinoma Tissue, C4724 Representative an Immunohistochemical Results of RBFOX1 of 82 Years Old Female Patient in Colon Adenocarcinoma Tissue. (<https://www.proteinatlas.org/search/RBFOX1>). B. Immunohistochemical Results of RBFOX1 in Two High-Throughput Pathological Tissue Chips (Consisting of 69 Colorectal Cancer Tissues). C. A Normal Brain Sample was Used as a Positive Control., On the Left is a 100x Microscopic Image of the Brain, And on the Right is a 200x Magnified Image of a Localized Area on the Left. D. Immunohistochemical Results of RBFOX1 in Intestinal Tissue from a Case of slow Transit Constipation Undergoing Total Colectomy. E. Immunohistochemical Results of RBFOX1 in Three Right-Sided Colorectal Cancers and Adjacent Tissues. N Means Normal, C Means Cancer.

resulting from genetic mutations, epigenetic alterations, or chromosomal instability. This critical distinction requires further validation through comprehensive cellular experiments and animal models to elucidate the precise mechanistic role of RBFOX1 in colorectal tumorigenesis.

Conclusion

In the right-sided colon and tissues with high microsatellite instability, RBFOX1 shows copy number loss and low mRNA expression. This characteristic is closely related to BRAF gene mutation, and the protein of RBFOX1 is absent in some colorectal cancer tissues.

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Statements and Declarations

Ethical Considerations

The use of all these samples has been approved by the ethics committee of Wuhan University (Approval No. 2021061).

Consent to Participate

Informed consent was obtained from all individual participants included in the study.

Author Contributions/CRedit

Jian Li and youheng wang (co-first Author) : Conceptualization, Methodology, Software, Investigation, Formal Analysis, Writing - Original Draft; Jian Li (co-first Author) and xu jian : Data Curation, Writing - Original Draft; Experiment; ding zhao: Resources, Supervision; Min Niu and liu yi: Software, Validation. Songlin Wan and Guangchun Li: Visualization, Writing - Review & Editing. Qun Qian (Corresponding Author): Conceptualization, Funding Acquisition, Resources, Supervision, Daojiang Li (Corresponding Author): Writing - Review & Editing.

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Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Supplemental Material

Supplemental material for this article is available online.

References

1. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal cancer. *Lancet (London, England)*. 2019;394(10207):1467-1480. doi:10.1016/s0140-6736(19)32319-0
2. Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer*. 2017;17(2):79-92. doi:10.1038/nrc.2016.126
3. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med*. 2015;21(11):1350-1356. doi:10.1038/nm.3967
4. Matano M, Date S, Shimokawa M, et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat Med*. 2015;21(3):256-262. doi:10.1038/nm.3802
5. Drost J, van Jaarsveld RH, Ponsioen B, et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature*. 2015;521(7550):43-47. doi:10.1038/nature14415
6. Li D, Lin C, Li N, et al. PLAGL2 and POFUT1 are regulated by an evolutionarily conserved bidirectional promoter and are collaboratively involved in colorectal cancer by maintaining stemness. *EBioMedicine*. 2019;45(7):124-138. doi:10.1016/j.ebiom.2019.06.051
7. Li D, Lin C, Chen M, et al. Comprehensive bioinformatics analysis of the characterization and determination underlying mechanisms of over-expression and co-expression of genes residing on 20q in colorectal cancer. *Oncotarget*. 2017;8(45):78642-78659. doi:10.18632/oncotarget.20204
8. Zhang B, Yao K, Zhou E, Zhang L, Cheng C. Chr20q amplification defines a distinct molecular subtype of microsatellite stable colorectal cancer. *Cancer Res*. 2021;81(8):1977-1987. doi:10.1158/0008-5472.Can-20-4009
9. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):11. doi:10.1126/scisignal.2004088
10. Tate JG, Bamford S, Jubb HC, et al. COSMIC: The catalogue of somatic mutations in cancer. *Nucleic Acids Res*. 2019;47(D1):D941-d947. doi:10.1093/nar/gky1015
11. Mermel CH, Schumacher SE, Hill B, Meyerson ML, Beroukhim R, Getz G. GISTIC2.0 Facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol*. 2011;12(4):R41. doi:10.1186/gb-2011-12-4-r41
12. Li T, Fu J, Zeng Z, et al. TIMER2.0 For analysis of tumor-infiltrating immune cells. *Nucleic Acids Res*. 2020;48(W1):W509-w514. doi:10.1093/nar/gkaa407
13. Niu B, Ye K, Zhang Q, et al. MSIsensor: Microsatellite instability detection using paired tumor-normal sequence data. *Bioinformatics (Oxford, England)*. 2014;30(7):1015-1016. doi:10.1093/bioinformatics/btt755
14. Li L, Feng Q, Wang X. PreMSIm: An R package for predicting microsatellite instability from the expression profiling of a gene

- panel in cancer. *Comput Struct Biotechnol J*. 2020;18(1): 668-675. doi:10.1016/j.csbj.2020.03.007
15. Thul PJ, Åkesson L, Wiking M, et al. A subcellular map of the human proteome. *Science (New York, NY)*. 2017;356(6340): 1-12. doi:10.1126/science.aal3321
 16. Martin CL, Duvall JA, Ilkin Y, et al. Cytogenetic and molecular characterization of A2BP1/FOX1 as a candidate gene for autism. *Am J Med Genet Part B: Neuropsychiatr Genet*. 2007;144b(7): 869-876. doi:10.1002/ajmg.b.30530
 17. Barnby G, Abbott A, Sykes N, et al. Candidate-gene screening and association analysis at the autism-susceptibility locus on chromosome 16p: Evidence of association at GRIN2A and ABAT. *Am J Hum Genet*. 2005;76(6):950-966. doi:10.1086/430454
 18. Bhalla K, Phillips HA, Crawford J, et al. The de novo chromosome 16 translocations of two patients with abnormal phenotypes (mental retardation and epilepsy) disrupt the A2BP1 gene. *J Hum Genet*. 2004;49(6):308-311. doi:10.1007/s10038-004-0145-4
 19. Lal D, Trucks H, Møller RS, et al. Rare exonic deletions of the RBFOX1 gene increase risk of idiopathic generalized epilepsy. *Epilepsia*. 2013;54(2):265-271. doi:10.1111/epi.12084
 20. Shibata H, Huynh DP, Pulst SM. A novel protein with RNA-binding motifs interacts with ataxin-2. *Hum Mol Genet*. 2000;9(9):1303-1313. doi:10.1093/hmg/9.9.1303
 21. Raghavan NS, Dumitrescu L, Mormino E, et al. Association between common variants in RBFOX1, an RNA-binding protein, and brain amyloidosis in early and preclinical Alzheimer disease. *JAMA Neurol*. 2020;77(10):1288-1298. doi:10.1001/jamaneurol.2020.1760
 22. Sengupta N, Yau C, Sakthianandeswaren A, et al. Analysis of colorectal cancers in British Bangladeshi identifies early onset, frequent mucinous histotype and a high prevalence of RBFOX1 deletion. *Mol Cancer*. 2013;12(1):1-11. doi:10.1186/1476-4598-12-1
 23. Hu J, Ho AL, Yuan L, et al. From the cover: Neutralization of terminal differentiation in gliomagenesis. *Proc Natl Acad Sci USA*. 2013;110(36):14520-7. doi:10.1073/pnas.1308610110
 24. Li J, Choi PS, Chaffer CL, et al. An alternative splicing switch in FLNB promotes the mesenchymal cell state in human breast cancer. *eLife*. 2018;7:e37184:1-28. doi:10.7554/eLife.37184
 25. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487(7407):330-337. doi:10.1038/nature11252
 26. Voutsadakis IA. Chromosome 20q11.21 amplifications in colorectal cancer. *Cancer Genomics Proteomics*. 2021;18(3 Suppl): 487-496. doi:10.21873/cgp.20274
 27. Carvalho B, Postma C, Mongera S, et al. Multiple putative oncogenes at the chromosome 20q amplicon contribute to colorectal adenoma to carcinoma progression. *Gut*. 2009;58(1):79-89. doi:10.1136/gut.2007.143065
 28. Andersen CL, Lamy P, Thorsen K, et al. Frequent genomic loss at chr16p13.2 is associated with poor prognosis in colorectal cancer. *Int J Cancer*. 2011;129(8):1848-1858. doi:10.1002/ijc.25841
 29. Mampaey E, Fieuw A, Van Laethem T, et al. Focus on 16p13.3 locus in colon cancer. *PLoS One*. 2015;10(7):e0131421. doi:10.1371/journal.pone.0131421
 30. Zhang B, Wang J, Wang X, et al. Proteogenomic characterization of human colon and rectal cancer. *Nature*. 2014;513(7518): 382-387. doi:10.1038/nature13438
 31. Grothey A, Fakih M, Tabernero J. Management of BRAF-mutant metastatic colorectal cancer: A review of treatment options and evidence-based guidelines. *Ann Oncol*. 2021;32(8):959-967. doi:10.1016/j.annonc.2021.03.206
 32. Tomassoni-Ardori F, Fulgenzi G, Becker J, et al. Rbfox1 up-regulation impairs BDNF-dependent hippocampal LTP by dysregulating TrkB isoform expression levels. *eLife*. 2019;8: e49673:1-28. doi:10.7554/eLife.49673