The New Role of HNF1A-AS1/miR-214/INHBA Signaling Axis in Colorectal Cancer

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Abstract
Background: Colorectal cancer (CRC) is the third most common cancer and one of the leading causes of death worldwide. Seriously threatens human life and health. Previous studies have identified that inhibin α (INHBA) could induce tumorgenesis and progression of CRC through the regulation of the TGF-β/Smad signal axis. The abnormal expression of INHBA is related to the poor prognosis of patients. The aim of this study was to identify the molecular mechanism of HNF1A-AS1 and miR-214 regulating INHBA and carcinogenesis through bioinformatics combined with experiments. Methods: The expression of HNF1A-AS1, miRNA-214-5p, INHBA in pan-cancer and CRC were investigated in the Cancer Genome Atlas (TCGA). The correlation between HNF1A-AS1 and immune-related genes or miRNAs was explored via the Gene Expression Profiling Interactive Analysis (GEPIA) and volcano plots, respectively. The association between HNF1A-AS1 and differentially expressed miRNAs was constructed by TargetScan. The miRDB, miRWalk, and TargetScan databases were utilized to predict the target genes of hsa-miR-214. The expression of INHBA in tissues and cell lines of CRC was examined by RT-qPCR and western blot assay. Results: The INHBA and HNF1A-AS1 expressions were increased in Colon adenocarcinoma (COAD) and Rectum adenocarcinoma (READ) of the TCGA database. Hsa-miR-214 was relatively less expressed in CRC tissues compared with para-cancer tissues. The expression of HNF1A-AS1 was negatively correlated with hsa-miR-214. INHBA was one of the target genes of hsa-miR-214 based on miRDB, miRWalk, and TargetScan databases. The specific binding sites of INHBA-3’UTR and miR-214-5p were identified by starBase. The expression level of INHBA was positively correlated with the T stage of tumor and negatively correlated with overall survival (OS) and disease-free survival (DFS) in CRC patients. The results of RT-qPCR and western blot indicated that the expression of INHBA in tissues and cell lines in CRC was higher than those in para-carcinoma tissues and normal colon cell lines, respectively. Conclusions: These findings suggested that HNF1A-AS1 and miRNA-214-5p were key upstream non-coding RNAs of INHBA. The HNF1A-AS1/miR-214/INHBA signal axis plays a significant role in the tumorgenesis and progression of CRC. By interfering with HNF1A-AS1 and INHBA genes on HT29 and SW480 cells, it was found that HNF1A-AS1 and INHBA genes may be important target genes in CRC.

Keywords: colorectal cancer; HNF1A-AS1; miR-214; INHBA; TGF-β/Smad

1. Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies. In 2020, the incidence of CRC ranked third and the mortality rate ranked second [1]. In addition, CRC is the third most common cancer in males and the second most common in females, with approximately 1.9 million new cases and 0.9 million deaths in 2020 worldwide [2]. CRC is a heterogeneous disease, regulated by numerous factors and intricate biological process, and its occurrence and developmental mechanism remains to be elucidated [3]. In recent years, epigenetic and transcriptional changes have attracted the attention of researchers due to their significant role in tumor development [4,5].
MiRNA is a noncoding single-stranded RNA (19–25 nt) derived from endogenous hairpin structure transcripts, and is a noncoding small molecular RNA. MiRNAs can induce target gene mRNA degradation or target gene translation inhibition through the 3′ UTR region or coding region specific base pairing combination, thus regulating target gene expression at the post-transcriptional level [6,7]. Studies have shown that miRNAs play a crucial role in tumor growth, apoptosis, invasion and metastasis [8]. They restrain the expression of target genes by combining with the translation of target genes, and then participate in the regulation of 30% human protein expression, tumorigenesis, development and metastasis [9]. Long noncoding RNA (LncRNA) also has an important role in the research of human tumors. As a competitive endogenous RNA (ceRNA), LncRNA performs a vital role in tumorigenesis by binding miRNAs to regulate the expression of target genes [10,11].

Inhibin is a homodimer or heterodimer consisting of α, βA and βB subunits, which are called inhibin α (INHA), inhibin βA (INHBA), and inhibin βB (INHBB) subunit gene [12]. INHBA gene is located on chromosome 7p15-p14 and consists of two exons [12]. As a member of the transforming growth factor beta (TGF-β) superfamily, INHBA gene participates in the occurrence and development of tumors by regulating the TGF-β/Smad signaling pathway. Abnormal expression of the INHBA gene is associated with poor prognosis of reproductive cancers [13]. Previous researchers have demonstrated that the INHBA gene also promotes the invasion and metastasis of cancers by inducing epithelial-mesenchymal transition (EMT), promoting angiogenesis and changing epigenetic mechanisms [14].

Based on the transcriptome data of 473 colon cancer tissues and 41 adjacent tissues collected in the cancer genome atlas (TCGA) database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), our research team found that INHBA was highly expressed in cancer tissues, and the expression of INHBA significantly positively correlated with the T stage of tumor, that is, the higher the expression level of INHBA, the later the T stage of patients. The survival curve analysis of gene expression profiling the interactive analysis (GEPIA) database (http://gepi.a.cancer-pku.cn/) demonstrated that the expression level of INHBA was negatively correlated with the survival rate of colon cancer patients, that is, the higher the expression level of INHBA, the lower the survival rate of colon cancer patients. RT-qPCR and western blot tests were performed on 17 clinical tumor tissues and para-cancerous tissues. The results showed that the mRNA and protein levels of INHBA were highly expressed in tumors. In addition, immunohistochemical (IHC) staining also demonstrated that INHBA protein was brownish yellow, was principally located in the cytoplasm, and had high expression of INHBA in cancer tissue. LncRNA HNF1A antisense RNA 1 (HNF1A-AS1) is a 2.455 kb LncRNA located on chromosome 12q24.31. It is transcribed from the opposite strand of the HNF1A gene [15]. Previous studies showed that HNF1A-AS1 and mir-214 were closely related to the progression of CRC, and bioinformatics confirmed that mir-214 specifically targets the 3′UTR of the INHBA gene. Further analysis indicated that the expression of HNF1A-AS1 and mir-214 was consistent in the TCGA database and experiments. In addition, previous studies have shown that the INHBA gene can regulate the TGF-β/Smad signaling pathway and promote the occurrence and development of tumors [16]. Therefore, we hypothesized that mir-214 regulates the activity of the TGF-β/Smad signal axis by targeting INHBA, thereby inhibiting the proliferation and metastasis of CRC.

2. Materials and Methods

2.1 Clinical Samples Collection

We collected 60 pairs of CRC tumor and para-cancer tissues from the Yunnan Cancer Hospital/The Third Affiliated Hospital of Kunming Medical University. The age of the CRC patients ranged from 46 to 69 years, including 31 males and 29 females. Baseline demographic and clinical characteristics of all 60 CRC patients were collected. All tumors and adjacent tissues removed from the patient were immediately placed in liquid nitrogen and transported to the laboratory within 2 hours for storage in a ~80 °C degree refrigerator. Written informed consent was obtained from all participants prior to their enrollment in the study. The protocols for the present study were approved by the Medical Institutional and Clinical Research Ethics Committee of the Yunnan Cancer Hospital, the Third Affiliated Hospital of Kunming Medical University (refers to the same entity) in accordance with the “Declaration of Helsinki” on the ethical principles for medical research involving human subjects.

2.2 Analysis of HNF1A-AS1 in Diverse Human Tumor

Previous studies have demonstrated that HNF1A-AS1 is a potential long noncoding RNA which regulates tumor progression. Using the TCGA database, we analyzed the expression of HNF1A-AS1 in 33 different tumors and adjacent tissues. In addition, we analyzed the expression of HNF1A-AS1 in colon cancer and rectal cancer tissues and corresponding para-cancer tissues and its association with overall survival (OS). We further verified differences in HNF1A-AS1 expression in colon cancer and rectal cancer in relative para-cancer tissues in the two databases.

2.3 Differential Expression of HNF1A-AS1/miRNAs

First, we used the “limma 2.11” R package to analyze the differences in HNF1A-AS1 expression in 477 tumors and 41 neighboring tissues in the TCGA database. Then, we analyzed HNF1A-AS1 in 125 immune related genes related to the distribution in the tumor and nearby tissue. Differential screening was set at a threshold for |log2 fold
change (FC) > 1 and a p value < 0.05, using a screening of the volcano map. Finally, to determine the correlation between HNF1A-ASI and differentially expressed miRNAs, TargetScan was used to predict and construct the correlation.

2.4 Gene Ontology (GO) Analysis

To explore the potential functions of 125 immune-related genes associated with HNF1A-ASI, GO (is an internationally standardized classification system of gene functions that provides a dynamically updated and controlled vocabulary to comprehensively describe the properties of genes and gene products in an organism) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the R package ‘clusterprofiler’ R package. In the analysis results, pathways or functions with a p value < 0.05 were considered to be significantly enriched.

2.5 Cell Culture and Cell Transfection

The human normal colon epithelial cell lines (NCM460) and the human colon cancer cell lines (HCT116, HT29, SW480, SW620, and LOVO) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The NCM460 and HCT116 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640; MeilunBio, Dalian, China) and Dulbecco’s modified Eagle’s medium (DMEM; Procell, Wuhan, China). The HT29 and SW480 cells were cultured in L15 media (Leibovitz’s L-15; Procell), the SW620 and LOVO cells were cultured in Fl1K media (Ham’s F-12K; Procell). Media were supplemented with 10% fetal bovine serum (FBS; Procell) and 100 µg/mL penicillin-streptomycin (Procell). Cells were grown at 37 °C incubator (FBS; Procell) and 100 µg/mL penicillin-streptomycin (Procell). DMEM or L15 media containing 15% FBS (Procell). Media were supplemented with 10% fetal bovine serum (FBS; Procell) and 100 µg/mL penicillin-streptomycin solution (Procell). Cells were grown at 37 °C incubator with 5% CO₂/95% air. This procedure was also used for the preparation and transfection of HNF1A-ASI shRNA and INHBA shRNA.

Using Lenti-Pac™ HIV Expression Packaging Kit (GeneCopoeia, Guangzhou, China), the interference plasmid and over-expression plasmid were transfected into 293T cells with DNA-lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA, USA) The complete medium was changed after transfection. After 48 h of incubation, the culture supernatant rich in lentivirus particles was collected and stored at −80 °C. Following polybrene™ (Merck KGaA, Darmstadt, Germany)-assisted HNF1A-ASI interference of lentivirus for 72 h, 1 µg/mL puromycin was used to screen the transfected positive cells. The medium was changed every 2 days and screening with complete medium was continued for 2 weeks to expand the cell culture. All cell lines were genotyped for identity by Shanghai Biowing Applied Biotechnology Co., Ltd. Mycoplasma Removal Agent (0.02ug /ml,C0288M, Biyuntian, Shanghai, China) was added during the experiment. The cell morphology was observed under the microscope, which was similar to that of the ATCC cell bank. STR identification was performed on the experimental cells.

2.6 3-(4,5)-dimethylthiazol-2-yl)-3,5-di-phenyltetrazoliumromide (MTT) Assay

Cell viability was demonstrated by a MTT kit (Solarbio, Beijing, China). After performing various treatments, HT29 and SW480 cells and their transfection with HNF1A-ASI shRNA and INHBA shRNA were cultured in 96-well plates for 24, 48, 72 and 96 h, respectively. Fresh media were mixed with MTT solution and serially added into culture plates. 4 h later, dimethyl sulfoxide (Sigma, St. Louis, MO, USA) was added into wells to dissolve formazan. Samples were assessed by microplate reader (Thermo Fisher, New York, NY, USA), and results were obtained by analyzing the output of the wavelength at 490 nm.

2.7 Flow Cytometry Analysis

Cell apoptosis was determined by an Annexin V-fluorescein isothiocyanate (Annexin V-PE; Procell) detection kit (Solarbio). Briefly, cultured cells were digested with trypsin (Thermo Fisher), and suspended in binding buffer (Solarbio). Annexin V-PE (Solarbio) and 7-aminoactinomycin D (7-AAD; Solarbio) were then serially incubated with cells in the dark. Finally, samples were analyzed with a flow cytometer (Thermo Fisher).

2.8 Cell Proliferation Ability Test

In preparation of cell suspension, the cells were digested with 0.25% trypsin and collected, and the cells were resuspended and counted with culture supernatant. The cells were seeded into 96-well plates by 4 × 10⁴ cells, with 5 replicates in each group. A total of four 96-well plates were seeded and returned to the incubator for further culture (measured once at 0 h, 24 h, 48 h, 72 h and 96 h, respectively). CCK8 reagent was diluted with basal medium at 10:1. The 96-well plate was removed to remove the cell supernatant in the culture plate, and 100 µL of diluted CCK8 solution was added to each well, and care was taken not to form bubbles in the wells. A blank control well was set, and 100 µL of diluted CCK8 solution was added to the other 3 wells. The samples were returned to the incubator and incubated for 2 h in darkness. The OD value of each well in the 96-well plate was detected at 450 nm with a microplate reader.

2.9 Transwell Invasion Assay

The invasion of HT29 and SW480 cells and their transfection with HNF1A-ASI shRNA and INHBA shRNA, respectively, was investigated with transwell chambers with Matrigel (Corning, Madison, NY, USA). Cells were seeded in the upper chambers with FBS-free DMEM or L15 media (Procell). DMEM or L15 media containing 15% FBS (Procell) were added into the lower chambers. After 24 h...
of culture, the cell supernatant was discarded and cells were singly incubated with methanol (Sigma) as well as crystal violet (Sigma). Results were analyzed by counting the cell numbers in the lower chambers under a microscope (Nikon, Tokyo, Japan) at a 100× magnification.

2.10 Association between HNF1A-AS1 and Differential miRNAs

The relative expression of miRNAs, which is tightly linked with HNF1A-AS1, was detected by RT-qPCR in clinical CRC and para-cancer tissues. Subsequently, the association between differentially expressed miRNAs and HNF1A-AS1 was analyzed.

2.11 Target Gene Analysis of hsa-miR-214

The miRDB, miRWalk, and TargetScan databases were utilized to predict the target genes of hsa-miR-214. RT-qPCR was used to detect the relative expression level of target genes located in the overlapping region. The relative expression levels of INHBA in CRC and para-cancer tissues were analyzed from the TCGA database. In addition, the expression of INHBA at different stages of CRC and its association with disease-free survival (DFS) and OS were analyzed.

2.12 qRT-PCR

Samples were homogenized with Trizol reagent (Takara, Kyoto, Japan), and total RNA was extracted according to the Trizol kit (Takara) instructions. After quantification by NanoDrop 2000 (Takara), 200 ng total RNA was utilized by the ReverTra Ace qPCR RT Kit (Takara) for reverse transcription according to the instructions. THUNDERBIRD SYBR® qPCR Mix (Takara) was used for quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) analysis. CFX96 Touch Real-Time PCR Detection System (Bio-RAD, Hercules, CA, USA) was used for RT-qPCR. GAPDH was used as an internal reference when determining the contents. Corresponding primer sequences are listed in Table 1.

2.13 Western Blotting

Cell lysis buffer solution was added to the sample, and total protein extract was obtained after repeated suspension and centrifugation at 4 degrees. Protein concentration in the extract was detected by the Bradford method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein electrophoresis, followed by 300 mA constant current for 1 h to transfer the protein onto the PVDF membrane. After sealing, the primary antibody was added and shaken overnight at 4 °C. After washing the membrane, a secondary antibody was added and incubated at 37 °C for 2 h. Then luminous solution was and tablet pressing, exposure and fixing were performed. ImageJ (version 1.8.0, LOCI, University of Wisconsin, Madison, WI, USA) analysis was used to obtain the images and determine the integral optical density of protein bands. GAPDH protein was selected as the internal reference.

2.14 Immunohistochemistry Assay

IHC assay was performed according to the previous process. Briefly, paraffin sections were placed at 60 °C for 2 h, then washed with dimethylbenzene solution, ethanol, and TBS in sequence. After the antigen was retrieved with citrate buffer solution, endogenous peroxidase was blocked by 1% hydrogen peroxide. Tissues were incubated with primary antibody overnight at 4 °C. Subsequently, the sections were incubated with HRP-labeled goat anti-rabbit immunoglobulin G (1:100; Abcam). Sections were visualized by diaminobenzidine. The sections were then counterstained with hematoxylin and washed by dimethylbenzene solution and ethanol. Finally, the sections were sealed with neutral balsam. The results of the immunohistochemical staining were analyzed with an optical microscope.

2.15 Statistical Analysis

SPSS 23.0 software (IBM Corp., Armonk, NY, USA) was used for data analysis, and graph pad prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical mapping. The measured data was represented by mean ± standard deviation (± s). If the data presented normal distribution, a paired sample t-test was used for comparison between two groups, and one-way analysis of variance was used for comparison between multiple groups. If the data was not normal, non-parametric testing was used. Counting data were tested by $\chi^2$ test. $p < 0.05$ was considered statistically significant.

### Table 1. The primers using in qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>H-mir-214-3p</td>
<td>ACAGCAGGCACACAGACAGCAGT</td>
</tr>
<tr>
<td>H-mir-1252-3P</td>
<td>CAAATGAGCTTTAATTTCTTTTT</td>
</tr>
<tr>
<td>H-mir-7151-3P</td>
<td>CTACAGGCTGGGATGGCGGCTCA</td>
</tr>
<tr>
<td>H-mir-3907</td>
<td>AGGTGCTCGGACGCTGGCGCAGT</td>
</tr>
<tr>
<td>H-mir-3934-3P</td>
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<tr>
<td>H-mir-4306</td>
<td>TGGAGAGAAAGGCAGT</td>
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<tr>
<td>H-mir-3665</td>
<td>AGCGAGGCGGGGCGGCCGG</td>
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<tr>
<td>H-mir-1303</td>
<td>TTTAGAGAAGGGGTCATTGTCCT</td>
</tr>
<tr>
<td>H-mir-4668-5P</td>
<td>GAAAATCCTTTTTGTTTTCACCA</td>
</tr>
<tr>
<td>H-mir-10527-5P</td>
<td>AAAGCAAAATGGTGGGAGAACGGC</td>
</tr>
<tr>
<td>miR-Reverse primer</td>
<td>GTCGTATCCAGTGAGCGGT</td>
</tr>
<tr>
<td>U6 F</td>
<td>CTCGCTTCGCAGCGACACA</td>
</tr>
<tr>
<td>U6 R</td>
<td>AACGCTTCAGCAATTTTCCG</td>
</tr>
<tr>
<td>HNF1A-AS1 F</td>
<td>TGATGCTGTTCCTCTTACCC</td>
</tr>
<tr>
<td>HNF1A-AS1 R</td>
<td>GAGTTCTGTTCTTCTGCCC</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>CCCCATCACATTCCTCCAGG</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>CATACGGCCACAGTTCCTCC</td>
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</table>

qRT-PCR, quantitative Reverse Transcription Polymerase Chain Reaction.
Fig. 1. LncRNA HNF1A-AS1 is up-regulated and associated with OS. (A,B) The expression of HNF1A-AS1 in disparate types of cancers were exhibited with red dots and in para-cancer is shown with green dots. Data derived from TCGA database portal in GEPIA. (B) The horizontal axis shows the type of tumor and the height of the bar represents the median expression of certain tumor types or normal tissue. (C) Kaplan-Meier analysis of colon cancer patients with high (n = 134) or low (n = 135) HNF1A-AS1 levels. Statistical analysis was performed by log-rank test. (D) Kaplan-Meier analysis of rectal cancer patients with high (n = 45) or low (n = 45) HNF1A-AS1 levels. Statistical analysis was performed by log-rank test. (E) The expression of HNF1A-AS1 is up-regulated in colon cancer compared with para-cancer tissues (*p < 0.05). (F) The expression of HNF1A-AS1 is up-regulated in rectal cancer compared with the para-cancer tissues (*p < 0.05). The triangle symbol highlights the two genes. The triangle symbol highlights the two tumor; OS, overall survival; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; HNF1A-AS1, LncRNA HNF1A antisense RNA 1.

3. Results

3.1 HNF1A-AS1 is Highly Expressed in Colon Cancer and Rectal Cancer Tissues Compared with Para-Cancer Tissues

GEPIA production plot analysis of LncRNA HNF1A-AS1 gene expression in multiple cancer types and paired normal samples was performed, with each dot representing a different tumor or normal sample (Fig. 1A). It can be seen from the TCGA database that HNF1A-AS1 is highly expressed in Colon adenocarcinoma (COAD) and Rectum adenocarcinoma (READ) (Fig. 1). The expression level of HNF1A-AS1 in colon and rectal cancer is correlated with OS (Fig. 1C,D). In addition, we found that in the database of colon cancer and rectal cancer, HNF1A-AS1 was highly expressed in cancer tissues compared with para-cancer tissues, and the difference was statistically significant (*p < 0.05) (Fig. 1E,F).

3.2 HNF1A-AS1 Related miRNAs Analysis

The hierarchical cluster analysis showed that the expression of hnf1a-as1 target genes was different in 477 tumors and 41 neighboring tissues (Fig. 2A). Similarly, there were differences in the expression of 125 immune-related genes associated with HNF1A-AS1 (Fig. 2B). With the cut-off |log2 (FoldChange)| ≥1 and p value < 0.05, compared with the control, we obtained 2027 up-regulated genes and 1404 down-regulated genes (Fig. 2C). In addition, based on TargetScan data, we found 65 miRNAs that may bind to HNF1A-AS1 (Fig. 2D). GO and KEGG analysis of 125 genes are shown related to immunity including CD4-positive, alpha-beta T cell differentiation involved in immune response, T-helper 17 type immune response, the intestinal immune network for IgA production (Fig. 3A,B).

3.3 MiR-214 was Low Expressed in CRC Compared with Para-Cancer Tissues

RT-qPCR results demonstrated that compared with para-cancer tissues, hsa-miR-1252-3p, hsa-miR-214, hsa-miR-3907, hsa-miR-4688-5p, hsa-miR-10527-5p were less expressed (*p < 0.05), while HNF1A-AS1 was highly expressed in the CRC tissues (*p < 0.05) (Fig. 4A). In addition, correlation analysis was conducted between HNF1A-
AS1 and the expression of hsa-miR-1252-3p, hsa-miR-214, hsa-miR-3907, hsa-miR-4688-5p, and hsa-miR-10527-5p. The results showed that HNF1A-AS1 was negatively correlated with the expression of hsa-miR-1252-3p, hsa-miR-214, hsa-miR-3907, hsa-miR-4688-5p and hsa-miR-10527-5p. The expression of HNF1A-AS1 was negatively correlated with that of hsa-miR-214, hsa-miR-4688-5p and hsa-miR-1252-3p (p < 0.05) (Fig. 4B,C,D,E,F).

3.4 The Target Gene INHBA of hsa-miR-214 is Highly Expressed in CRC

The miRDB, miRWalk, and TargetScan databases predicted four overlapping genes of miR-214 target genes, namely CXCR5, GFRA1, SNAP25 and INHBA (Fig. 5A). RT-qPCR results showed that compared with adjacent tissues, CXCR5, GFRA1, and SNAP25 genes were less expressed in CRC, while INHBA gene was highly expressed in CRC (p < 0.001) (Fig. 5B–E).

3.5 MiRNA-214-5p is Positively Correlated with INHBA in Colon Cancer

The specific bindings sites of INHBA-3'UTR and miR-214-5p were identified by starBase. The results indicated that INHBA might be the target gene of miR-214-5p, that is, miR-214-5p might regulate the expression of the INHBA gene through a post-transcriptional mode (Fig. 6A). Through enrichment analysis of the INHBA gene, it was found that miR-214-5p may be one of the key miRNAs regulated upstream from INHBA (Fig. 6B). Analysis of 450 colon cancer samples by TCGA database indicated that miR-214-5p is positively correlated with INHBA (Fig. 6C). The TCGA database was used to analyze the miRNAs data of 261 CRC tissues and normal control tissues. The results demonstrated that miR-214-5p was less expressed in tumor tissues compared with the adjacent tissues (p < 0.01) (Fig. 6D).
Fig. 3. GO annotation and KEGG pathway enrichment analysis. (A) A biological process rich in 125 genes related to immunity. (B) KEGG pathway enriched by 125 immune-related genes. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Fig. 4. Expression and correlation of HNF1A-AS1 and related miRNAs. (A) mRNA expression of HNF1A-AS1 and related miRNAs in tumor and normal tissues. (B) The correlation analysis between hsa-miR-1252-3p and HNF1A-AS1 expression in CRC tissues. (C) The correlation analysis between hsa-miR-214 and HNF1A-AS1 expression in CRC tissues. (D) The correlation analysis between hsa-miR-3907 and HNF1A-AS1 expression in CRC tissues. (E) The correlation analysis between hsa-miR-4668-5p and HNF1A-AS1 expression in CRC tissues. (F) The correlation analysis between hsa-miR-10527-3p and HNF1A-AS1 expression in CRC tissues (*p < 0.05, **p < 0.01, ***p < 0.001). R < 0.3, uncorrelated; p < 0.05, significant correlation.
3.6 The Expression of INHBA Gene is Associated with the Progression of CRC

According to the transcriptome data of 473 CRC and 41 adjacent tissues gathered in the TCGA database, it was found that INHBA was highly expressed in cancer tissues (Fig. 7A,B), and the expression level of INHBA was positively correlated with the T stage of tumor, that is, the higher the expression level of INHBA, the later the T stage of patients \((p < 0.05)\) (Fig. 7C). However, there was no correlation between the expression level of INHBA and the N stage, or M stage of the tumor \((p > 0.05)\) (Fig. 7D,E). The survival curve analysis of the GEPIA database demonstrated that there was a negative correlation between the expression level of INHBA and the survival rate of CRC patients, that is, the higher the expression level of INHBA, the lower the OS and DFS of CRC patients \((p < 0.05)\) (Fig. 7F,G). A prognostic risk score model of CRC was constructed based on the INHBA gene, and the area under the curve (AUC) was: 0.996 (95% CI: 0.993–1.000, \(p < 0.05\)), indicating that the INHBA gene has high accuracy in predicting the prognosis of CRC (Fig. 7H).

3.7 INHBA is Highly Expressed in CRC Based on Experimental Verification

The expression level of INHBA was detected by the immunohistochemical method in 60 pairs of CRC tissues and adjacent tissues, and the positive rate was analyzed by Image-Pro Plus scanning software. Compared with para-
### 3.8 Transfection and Biological Capacity of HT29 and SW480 Cells

The proliferation and invasion abilities of HT29 cells in HNF1A-AS1 shRNA ($p < 0.01$) and INHBA shRNA ($p < 0.01$) were both attenuated compared with those in the NC group (Fig. 9A,C), but the apoptosis was the opposite (Fig. 9E). The proliferation, invasion and apoptosis of SW480 cells in HNF1A-AS1 shRNA ($p < 0.01$) and INHBA shRNA ($p < 0.01$) were similar to those of HT29 (Fig. 9B,D,F). This indicates that HNF1A-AS1 and INHBA may be key target genes in CRC cell lines.

### 3.9 Patient Characteristics

A total of 60 pairs of tumor tissue samples from CRC patients meeting the inclusion and exclusion criteria were collected. The general baseline demographic and clinical data of patients are demonstrated in Table 2.

### 3.10 Relationship between Expression Level of INHBA and Clinicopathologic Features in CRC

$\chi^2$ test was used to analyze the correlation between the expression of INHBA protein and the clinicopathologic characteristics of 60 CRC patients, such as age, gender, tumor size, degree of differentiation, depth of invasion (T stage), lymph node metastasis (N stage), distant metastasis (M stage), TNM stage, vascular invasion, nerve invasion, expression status of mismatch repair protein (MMR), and tumor location. The results showed that the expression of INHBA was significantly correlated with the degree of differentiation, depth of invasion, lymph node metastasis, distant metastasis, TNM stage, MMR status, and vascular invasion ($p < 0.05$), but was not significantly correlated with gender, age, tumor size, tumor location and nerve invasion ($p > 0.05$), as shown in Table 3.
Fig. 7. Bioinformatics analysis of the expression of \textit{INHBA} in CRC and its correlation with the prognosis. (A,B) The expression of \textit{INHBA} in CRC and adjacent tissues was analyzed by TCGA database. (C) The expression of \textit{INHBA} in different T stages of CRC was analyzed by TCGA database. (D) The expression of \textit{INHBA} in different N stages of CRC was analyzed by TCGA database. (E) The expression of \textit{INHBA} in different M stages of CRC was analyzed by TCGA database. (F) OS analysis corresponding to \textit{INHBA} expression in CRC patients in GEPIA database. (G) DFS analysis corresponding to \textit{INHBA} expression in CRC patients in GEPIA database. (H) Prognostic risk scoring model of CRC based on \textit{INHBA} gene (*\textit{p} < 0.05, **\textit{p} < 0.01, ***\textit{p} < 0.001, ****\textit{p} < 0.0001). DFS, Disease-free survival.

Fig. 8. Expression of \textit{INHBA} in CRC tissues and cell lines. (A,B) Positive rate in normal and tumor tissues detection by IHC. (C,D) Expression levels in normal and tumor tissues detected by western blot. (E,F) Expression levels in CRC cell lines NCM460, HCT116, HT29, SW480, SW620 and LOVO detected by western blot. (G) mRNA expression levels in CRC cell lines NCM460, HCT116, HT29, SW480, SW620 and LOVO detected by qRT-PCR. The protein expression was normalized against β-actin, and mRNA expression was normalized for GAPDH. Expressed as relative expression ratio. (\textit{p} < 0.05, **\textit{p} < 0.01, ***\textit{p} < 0.001, ****\textit{p} < 0.0001). IHC, immunohistochemical.

4. Discussion

Previous studies have shown that the \textit{INHBA} gene can participate in the occurrence and development of gastric
Fig. 9. Biological function of HNF1A-AS1 and INHBA in human CRC cell lines. (A,B) HT29 and SW480 cells and their transfection with HNF1A-AS1 shRNA and INHBA shRNA, respectively HT29 and SW480 cells and their transfection with HNF1A-AS1 shRNA and INHBA shRNA, respectively proliferation at 24, 48, 72 and 96 h. (C,D) Apoptosis detection by flow cytometry. (E,F) Transwell invasion assay detects cell invasion ability (\(**p < 0.01, ***p < 0.001, ****p < 0.0001\)).

cancer by regulating the TGF-\(\beta\)/Smad signaling pathway [16]. It can also promote the invasion and metastasis of pancreatic cancer, breast cancer, hepatocellular carcinoma and non-small cell lung cancer by inducing EMT [17,18], promoting angiogenesis [14] and changing epigenetics [19]. The TGF-\(\beta\)/Smad signaling pathway is a tumor inhibitory pathway mediated by the membrane serine/threonine kinase receptor. Its main components include the TGF-\(\beta\) Superfamily, TGF-\(\beta\) Receptors, Smad Protein Family. Its nuclear transcription regulators and abnormalities in any component of this pathway can lead to tumorigenesis [20]. A recent study found that the knockdown of INHBA can significantly inhibit the expression of the TGF-\(\beta\) signaling pathway-related proteins in CRC cells [21].

However, the role of the INHBA-mediated TGF-\(\beta\)/Smad signaling axis in the development and progression of CRC remains to be further studied. The TCGA database was used to analyze the expression of INHBA in CRC and adjacent tissues, and the results demonstrated that INHBA was highly expressed in cancer tissues, and the expression level of INHBA was positively correlated with the T stage of tumor, that is, the higher the level of INHBA, the higher the
and DFS in CRC patients. Therefore, we hypothesize that HBA expression level was negatively correlated with OS.

In addition, our study found that the higher the expression of INHBA in CRC cell lines (HT29 and SW480), highly expressed in CRC cell lines (HT29 and SW480), tissues were significantly higher than that in the control adjacent tissue (NCM460), confirmed that the expression level of INHBA in the CRC tissues was significantly higher than that in the control adjacent tissue. In addition, compared with normal intestinal epithelial cells (NCM460), INHBA protein levels were highly expressed in CRC cell lines (HT29 and SW480). In addition, our study found that the higher the expression level of INHBA in CRC patient tumor tissues, the worse degree of differentiation, the higher T stage, N stage, and M stage, the more likely they are to develop vascular invasion, and to exhibit pMMR status. It was also found that there was no significant difference in the expression level of INHBA in either the right or left colon. Finally, survival curve analysis of the GEPIA database demonstrated that INHBA expression level was negatively correlated with OS and DFS in CRC patients. Therefore, we hypothesize that INHBA, as a tumor suppressor gene, also promotes CRC progression by regulating the TGF-β/Smad signaling pathway, which will be further verified in subsequent studies.

Previous studies have confirmed that miR-214 can play a tumor suppressive role in endometrial cancer, breast cancer and other tumors through diverse molecular mechanisms [22,23]. Studies also suggested that miR-214 can be used as a crucial tumor suppressor in CRC [24]. For example, miR-214 can inhibit the proliferation and metastasis of CRC by targeting the PLAGL2-MYH9 axis [25], while miR-214 can also inhibit LIVIN and the NF-kB signaling pathway, which plays an anti-cancer role. Therefore, miR-214 may inhibit the proliferation and metastasis of CRC. MiRNAs data of 261 colon cancer tissue samples and control samples collected from the TCGA database were analyzed, and the results showed that miR-214 was less expressed in tumor tissues than in normal controls (p < 0.01). Other studies have found that miR-214 can inhibit TGF-β mediated activation of pancreatic stellate cells and the occurrence of pancreatic cancer [26], but whether miR-214 can inhibit the occurrence and development of CRC by down-regulating the activity of TGF-β/Smad signaling axis remains to be further verified. Bioinformatics enrichment analysis found that miR-214 may be one of the key miRNAs regulated upstream of INHBA, and it was demonstrated that INHBA-3’UTR specifically binds to miR-214.

LncRNA has been proven to regulate various biological processes such as cell proliferation, apoptosis, invasion and metastasis, which provides a new perspective for the diagnosis and treatment of cancer [27]. HNF1A-ASI is highly expressed in diverse tumors, promoting the proliferation of triple-negative breast cancer [28], hepatocellular carcinoma [29] and glioma [30]; proliferation, migration and invasion of osteosarcoma [31]; proliferation, apoptosis, chemoresistance of non-small-cell cancer (NSCLC) [32]; proliferation, migration, EMT of oral squamous cell carcinoma [33]; invasion, metastasis, angiogenesis and lymphangiogenesis of gastric cancer [34]; metastasis and invasion of bladder cancer [35]; and proliferation, migration and invasion of esophageal adenocarcinoma [36]. Our results from TCGA database and clinical samples showed that HNF1A-ASI was highly expressed in CRC. In addition, there was a negative correlation between the expression of HNF1A-ASI and miR-214 (p < 0.05).

### Table 2. The Baseline demographic and clinical characteristics of 60 CRC patients.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Sample size (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male 31, female 29</td>
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<tr>
<td>Age (years)</td>
<td>≥50 50, &lt;50 10</td>
</tr>
<tr>
<td>Tumor size</td>
<td>≥3 cm 53, &lt;3 cm 7</td>
</tr>
<tr>
<td>Tumor differentiation</td>
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<tr>
<td>Depth of invasion (T stage)</td>
<td>T1 + T2 18, T3 + T4 42</td>
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<tr>
<td>Lymph node metastasis (N stage)</td>
<td>Yes 30, No 30</td>
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<tr>
<td>Distant metastasis (M stage)</td>
<td>Yes 3, No 57</td>
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<tr>
<td>TNM staging</td>
<td>I + II 28, III + IV 32</td>
</tr>
<tr>
<td>Vascular infiltration</td>
<td>Yes 12, No 48</td>
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<tr>
<td>Nerve invasion</td>
<td>Yes 6, No 54</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Right semicolon 21, Left semicolon + rectum 39</td>
</tr>
<tr>
<td>Mismatch repair protein (MMR) status</td>
<td>dMMR 6, pMMR 54</td>
</tr>
</tbody>
</table>

TNM, tumor node metastasis; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair.

In conclusion, our preliminary results suggest that the HNF1A-ASI/miR-214/INHBA axis regulates the TGF-β/Smad signaling pathway, which may be a potential mechanism affecting CRC. Subsequently, we will conduct detailed verification of the regulatory relationship between HNF1A-ASI/miR-214/INHBA axis at the cellular and animal levels. Meanwhile, in vitro and in vivo response experiments were conducted to demonstrate whether the HNF1A-ASI/miR-214/INHBA axis affects the development of CRC.
Table 3. Relationship between INHBA expression level and clinicopathologic features of CRC.

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Sample size (n = 60)</th>
<th>Expression level of INHBA</th>
<th>$\chi^2$</th>
<th>$p$</th>
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<tr>
<td></td>
<td></td>
<td>High expression</td>
<td>Lower expression</td>
<td></td>
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<tr>
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<td>11</td>
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<tr>
<td></td>
<td>Female</td>
<td>29</td>
<td>13</td>
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<tr>
<td>Age (years)</td>
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<td>50</td>
<td>20</td>
<td>0.3536</td>
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<td></td>
<td>&lt;50</td>
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<tr>
<td></td>
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<tr>
<td>Tumor differentiation</td>
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<tr>
<td></td>
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<td>28</td>
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<tr>
<td></td>
<td>Low</td>
<td>27</td>
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<td></td>
<td>No</td>
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<tr>
<td>Nerve invasion</td>
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<td>1.151</td>
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<td></td>
<td>No</td>
<td>54</td>
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<tr>
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<tr>
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<td>pMMR</td>
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<td>20</td>
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</table>

by regulating the TGF-β/Smad signaling pathway. Our results will provide new prognostic markers and therapeutic targets for CRC.

Availability of Data and Materials


Author Contributions

XZ, YL, TW, and RD were responsible for study design, data acquisition, and analysis and were major contributors to writing the manuscript. RQ, GL, YZ, WX, ZS, and RY helped to perform the mining of biological information and the data analysis. XC, YR, CD, and JL helped to perform the collection of specimens and experimental verification. All authors contributed to the article and approved the submitted version. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study protocols were approved by the Medical Institutional and Clinical Research Ethics Committee of Yunnan Cancer Hospital, the Third Affiliated Hospital of Kunming Medical University (Approval number: KYCS2022033). All experiments were carried out in accordance with the “Declaration of Helsinki” on the ethical principles for medical research involving human subjects. Written informed consent was obtained from all participants prior to their enrollment of the study.
Acknowledgment
Not applicable.

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Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2811301.

References


