Interaction between C2ORF68 and HuR in human colorectal cancer

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Received April 26, 2018; Accepted October 31, 2018

DOI: 10.3892/or.2019.6973

Abstract. The detailed molecular mechanisms underlying the carcinogenesis of colorectal carcinoma (CRC) remain unknown. Therefore, the present study was designed to investigate the effect of the relationship between C2ORF68 and HuR in regards to the carcinogenesis of CRC. Immunohistochemistry, immunofluorescence, flow cytometry, Transwell migration and CCK-8 assays, co-immunoprecipitation, qRT-PCR and western blot analysis were performed. The results revealed that expression of C2ORF68 was significantly upregulated in the cytoplasm and nucleus in rectal cancer, and upregulation of the expression of C2ORF68 was associated with lymph node metastasis and pathological grade. C2ORF68 and HuR were found to be mainly localized in the nucleus in both SW480 and LoVo cells. In LoVo + c2orf68, SW480 - c2orf68 and LoVo + HuR, SW480 - HuR cells, the cell apoptosis rate was significantly decreased, cell proliferation rate was significantly increased, and the cell migration rate was only significantly increased in the LoVo + c2orf68, SW480 - c2orf68 and SW480 - HuR, the cell apoptosis rate was significantly increased. At the same time, cell proliferation and the cell migration rate were significantly decreased. The mRNA and protein expression levels of C2orf68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A were upregulated, while the expression of Bax was downregulated in LoVo + c2orf68 and LoVo + HuR cells. Expression levels of C2orf68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A were downregulated while Bax was upregulated in the SW480 - c2orf68, HuR, SW480 + c2orf68 and SW480 + HuR cells. In conclusion, it is suggested that c2orf68 is a potential carcinogenesis factor in rectal cancer. Furthermore, c2orf68 may have a synergistic effect with HuR in the onset and development of CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignant tumor and is the fourth leading cause of cancer-related death worldwide (1). It is largely diagnosed in individuals >50 years of age (2). CRC patients in the early stage [tumor-node-metastasis (TNM) stage I and II] present with a prolonged 5-year survival following surgical excision; the 5-year survival is up to 95% and 60-80% for stage I and II, respectively. However, existing therapies for CRC usually exhibit a limited effect on patient prognosis, and the 5-year survival of patients in stages III and IV can be as low as 35 and 10% (3). Research has shown that CRC incidence and mortality can be decreased significantly through screening programs, while CRC screening is only offered to very few individuals worldwide based on the CRC incidence rate, national economic level and medical security system (4). A previous study demonstrated that germline mutations enable next generation hereditary susceptibility to CRC accounting for 6-7%. In addition, mutations in DNA repair genes and signal transduction genes also contribute to the occurrence of CRC. In addition to inherited genetic mutations, environmental factors, such as the heavy consumption of alcohol, smoking habit, increased body fat and diets high in fat, salt and red and processed meat, also play important roles (5). However, more and more studies have shown that CRC displays accumulated defects in the activation of oncopgenes and the inactivation of tumor-suppressor genes (TSGs) (6). Except for classical CRC risk factors, such as: KRAS, TP53, APC and markers for microsatellite instability (MSI) (7,8), an impressive body of literature indicates that multiple factors such as hsa-miR-19a (9), PROK2 (10), B7-H3 (11), DSCC1 (12) and microRNAs (13) are involved in the occurrence of CRC. A recent study revealed that the dysbiosis of microbial communities in the human body are also associated with gastrointestinal tract cancer, such as gastric and colorectal cancer. Moreover, a hypothesis called ‘Alpha bug’ considers that some bacteria could alter the primary bacterial community and the remodeled bacterial community could promote CRC by strengthening the mucosal immune response (14). Although numerous studies have shown that the pathogenesis of CRC is multifactorial, the detailed mechanisms remain unclear at present.

C2ORF68, which belongs to the UPF0561 family, contains 166 amino acids, and the monoisotopic molecular weight is...
Clinicopathological parameters of the CRC patients are shown in Table I. The primary antibody (Ab) used for IHC was mouse monoclonal C2ORF68 Ab (1:200; BIO014915; Beacombio, Birmingham, UK). The expression level of C2ORF68 protein in rectal cancer tissues were scored by three independent examiners. The level of C2ORF68 staining pattern was scored according to four subgroups: i) Negative (-); ii) weak (+); iii) moderate (++); and iv) strong (+++).

Cell lines and cell culture. Human colon cancer cell lines, SW480 and LoVo [American Type Culture Collection (ATCC) Manassas, VA, USA], were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) which contained 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 IU/ml). The cells were grown at 37°C in a humidified atmosphere with 5% CO₂. The cells were harvested when they were in the exponential growth phase and then the experiments stated above were performed.

Immunoﬂuorescence (IF). For immunostaining, the SW480 and LoVo cells were ﬁxed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 15 min and blocked with 3% bovine serum albumin (BSA) for 30 min at room temperature. The treated cells were then incubated with mouse monoclonal anti-C2ORF68 (1:100) and rabbit polyclonal anti-HuR (dilution 1:100; cat. no. ab200342; Abcam, Cambridge, UK) overnight at 4°C, and ﬁnally incubated with FITC-labeled and TRITC-labeled secondary Ab for 30 min under the conditions of protection from light at room temperature. Each step was followed by two 5-min washes in PBS. The nuclei were counterstained using DAPI, and observed using an Olympus BX53 fluorescence microscope (Olympus, Hamburg, Germany).

siRNA selection and transient transfection. siRNAs for c2orf68 (siRNA sequences, 5’-CUAUGAAGUGCCUG UGAAUdTdT-3’ and 3’-dtdTCUUCUCCAAUCUACG AU-5’) and HuR (siRNA sequences, 5’-GGUUGGGUUAUC CCGGUU UdTdT-3’ and 3’-dtdTCCACGCAAUGG CCAA-5’) were designed and synthesized by RiboBio (Guangzhou, China). Using the blank and negative control groups, the transfection was performed with 100 nM of siRNA and Invitrogen™ Lipofectamine 2000™ (Thermo Fisher Scientiﬁc, Inc., Waltham, MA, USA) to induce the knockdown of c2orf68/HuR expression. After transfection for 6 h, the cells were respectively incubated in fresh DMEM for 48 h to detect the mRNA expression level and for 72 h to detect the protein expression level.

Strain, plasmid, plasmid extraction and overexpression. The c2orf68 gene was synthesized and inserted into the XhoI/EcoRI site of the pcDNA3.1 eukaryotic expression vector (Pharma Co., Shanghai, China), which was veriﬁed by restriction digestion followed by sequencing (Beijing Genomic Institute, Beijing, China) in our previous study (17). The microbial strain and plasmids used in the present study were Escherichia coli (E. coli), DH5α and the pcDNA3.1-c2orf68 eukaryotic expression vector. The plasmid extraction process was carried out by TIANprep Plasmid Mini kit introduction (cat. no. DP103-02; Tiangen Biotech Co., Ltd., Beijing, China).
The LoVo cells at a density of $3 \times 10^5$ cells/well in a 6-well plate transfected with 5 µl interference fragment or negative control (NC) vector using Lipofectamine 2000 which was then replaced with fresh growth medium after 6 h. Following culture for 48 h, the transfected LoVo cells were treated with 600 µg/ml of G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After 14 days, the monoclonal cells were cultured in the presence of 300 µg/ml of G418.

Co-immunoprecipitation (co-IP). For co-IP, pcDNA3.1-c2orf68 eukaryotic expression vector was transfected for 60 h in SW620 cells. Then the cells with overexpression of c2orf68 were lysed with mild lysis buffer containing several protease inhibitors for 30 min, and the cell lysates were separated by centrifugation at 15,000 x g for 20 min. Next, a modicum of cell lysates was reserved and utilized for western blot analysis. Next, the mouse anti-C2ORF68 Ab and rabbit anti-HuR Ab were added into the cell lysates for one night at 4°C. On the following day, 100 µl protein A+G were added into the compound and incubated for 4 h. Subsequently, the sediments were gathered and the beads were washed twice using mild lysis buffer. After that, the same volume of 2X SDS-PAGE loading buffer was used to elute the protein which was absorbed on sepharose beads. Finally, the protein was used for SDS-PAGE.

**Cell proliferation assay.** The cell proliferation assay was performed as previously described (19). Plates were read at an absorbance wavelength of 450 nm with the help of a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). Each transfection group had six replicates, and the experiment was repeated three times.

**Flow cytometry.** Flow cytometry was performed as previously described (19). Then, the results were analyzed by flow cytometry (FACSAria II Cell Sorter; BD Biosciences, Franklin Lakes, NJ, USA). Each transfection group had three replicates, and the experiment was repeated three times.

**Cell migration assay.** Cell migration assay was performed as previously described (19). Migrated cells were quantified by counting the stained cells under a microscope (Model 680; Bio-Rad Laboratories) at x200 magnification. For each well, five random fields were selected to determine the total number of migrated cells. The assay was performed in triplicate and repeated three times.

**qRT-PCR analysis.** qRT-PCR analysis was performed as previously described (19). The sequences of forward and reverse primers are shown in Table II. The amplification was performed on a Bio-Rad C1000 Touch Thermal Cycler.
LV et al: INTERACTION BETWEEN c2orf68 AND HuR

1921

The GAPDH gene was used as an endogenous control, and the ΔΔCq (20) method was used to quantify the data, and the experiment was repeated three times.

Western blot analysis. Western blotting was performed as previously described (19). The primary antibodies used for western blotting were as follows: C2ORF68 (cat. no. ab81363; Abcam, Cambridge, UK), Bcl-2 (cat. no. sc-492; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bax (cat. no. sc-623; Santa Cruz Biotechnology, Inc.), c-Myc (cat. no. ab32072; Abcam), (cat. no. ab200342; Abcam), cyclin D1 (cat. no. ab134175; Abcam), cyclin A (cat. no. ab181591; Abcam) and β-actin (cat. no. sc-8432; Santa Cruz Biotechnology, Inc.). All the primary antibodies used in this step at 1:1,000 dilution. Signals detection were performed through a Gel Imaging system (ProteinSimple, Santa Clara, CA, USA). Subsequent densitometry analysis were conducted using ImageJ (version 1.52g; National Institutes of Health, Bethesda, MD, USA).

Table II. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Location</th>
<th>Product length, bp</th>
</tr>
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<tbody>
<tr>
<td>c2orf68</td>
<td>F: GAAGAGTCGGTGAAACGAG</td>
<td>315-336</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>R: TACGCAACTTGAGGCTTCTC</td>
<td>483-462</td>
<td></td>
</tr>
<tr>
<td>HuR</td>
<td>F: ACCCAGGATGATGTTACGA</td>
<td>245-264</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>R: GCCCAAACCGAGAACAAT</td>
<td>369-349</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>F: AAGGCTAGGGTGTCTCAAG</td>
<td>172-192</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>R: CAAATGAAAGGGCGACAAC</td>
<td>349-328</td>
<td></td>
</tr>
<tr>
<td>Bcl2</td>
<td>F: GTTTTAGTTCTCTGGCTGTC</td>
<td>1-23</td>
<td>133</td>
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<tr>
<td></td>
<td>R: GAACCTTTTCATTTTGTTGG</td>
<td>649-627</td>
<td></td>
</tr>
<tr>
<td>c-Myc</td>
<td>F: TCAAGAGGGCAACACACAC</td>
<td>1,631-1,550</td>
<td>110</td>
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<tr>
<td></td>
<td>R: GGCCCTTTTCATTTTCCA</td>
<td>1,740-1,721</td>
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<tr>
<td>Cyclin D1</td>
<td>F: GTGGCCTCTAAGATGAGAGAG</td>
<td>534-555</td>
<td>169</td>
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<tr>
<td></td>
<td>R: GGAAGTGTCATGAATACGTG</td>
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<tr>
<td>Cyclin A</td>
<td>F: GTGCTCATGACCTTCAACA</td>
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<td>117</td>
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<tr>
<td></td>
<td>R: CTCTGAGGGGTGAGGAGGAG</td>
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<tr>
<td>GAPDH</td>
<td>F: GGAAGGTGAAAGGTCCGAGT</td>
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<tr>
<td></td>
<td>R: TGAGGTCATGAAGGGGTC</td>
<td>295-277</td>
<td></td>
</tr>
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F, forward; R, reverse.

Expression of C2ORF68 in the rectal cancer tissue microarray. The representative cytoplasmic staining of C2ORF68 in the rectal cancer tissue microarray is shown in Fig. 2A-C (magnification, ×400). Our previous study (15) demonstrated that C2ORF68 presents two different staining patterns, including nuclear staining and cytoplasmic staining. In line with the observation in IF, C2ORF68 is a predominantly nuclear protein, but cytoplasmic C2ORF68 localization may play a vital role in the occurrence of CRC. In the rectal cancer tissue microarray (Fig. 2A), C2ORF68 protein expression was detected in 95.56% (86/90) of the cancer samples. Among these, 4.44% (4/90), 16.67% (15/90), 45.56% (41/90) and
33.33% (30/90) of these cases exhibited negative (-), weak (+), moderate (+++) and strong (++++) C2ORF68 protein staining, respectively. In contrast, 5.56% (5/90), 25.56% (23/90), 58.89% (53/90) and 10% (9/90) of normal rectal specimens exhibited negative (-), weak (+), moderate (+++) and strong (++++) C2ORF68 protein staining, respectively (Fig. 2C). We selected five nonoverlapping views randomly from each image and calculated the mean optical density. Then, the difference in mean optical density between rectal cancer and adjacent normal tissue was analyzed by statistical analysis. It was shown that compared with the adjacent normal rectal tissues, the expression of C2ORF68 was significantly increased in the rectal cancer tissues (P<0.05). In addition, we also researched the relationship between the expression of C2ORF68 and clinical parameters. Significant associations were noted between C2ORF68 expression and pathological grade (P<0.05) and lymph node metastasis (P<0.05). However, the association between C2ORF68 expression and age, sex and TNM stage was not statistically significant.

Cellular localization of C2ORF68 and HuR in SW480 and LoVo cells. The results revealed that C2ORF68 and HuR were localized mainly in the nucleus in both SW480 and LoVo cells (Fig. 3A) (magnification, x100). BioGrid predicted that there is an interaction between C2ORF68 and HuR. It was shown that C2ORF68 interacts with HuR by Co-IP (Fig. 3B).

Cell apoptosis, cell proliferation and cell migration in LoVo+/-c2orf68, HuR and LoVo+/-c2orf68 cells. Following transfection for 24 h, flow cytometry was performed to analyze c2orf68 and HuR-induced cell cycle arrest and apoptosis in colon cancer cells. The cell apoptosis rate of LoVo+/-c2orf68, HuR and LoVo+/-c2orf68 cells was significantly decreased compared to that of the LoVo-NC and LoVo cells (Fig. 4B, P<0.05). There was no statistical significance noted between LoVo+/-c2orf68, HuR and LoVo+/-c2orf68, LoVo-NC and LoVo cells. Compared with the control group, LoVo+/-c2orf68 and LoVo+/-c2orf68, HuR cell proliferation was significantly increased (P<0.05); and the cell proliferation of LoVo+/-c2orf68 cells was statistically significantly different when compared with that of LoVo+/-c2orf68, HuR cells (Fig. 4C, P<0.05). Following transfection for 24 h, the number of migrated LoVo+/-c2orf68, HuR, LoVo+/-c2orf68, LoVo-NC and LoVo cells was 223±24, 379±33, 239±31 and 244±26, respectively. The number of migrated LoVo+/-c2orf68 cells was...
Figure 4. (A) Cell migration was observably increased in LoVo+c2orf68 cells. The number of migrated LoVo+c2orf68 cells was significantly higher than that of the LoVo-c2orf68, -HuR cells, while there was no observable difference between LoVo+c2orf68 and LoVo cells. (B) In the LoVo+c2orf68 and LoVo-c2orf68, -HuR cells, cell apoptosis was significantly decreased (P<0.05). There was no statistical significance noted between LoVo+c2orf68, -HuR and LoVo+c2orf68 cells. (C) In LoVo+c2orf68 and LoVo+c2orf68, -HuR cells, cell proliferation was increased. The cell proliferation of LoVo+c2orf68 cells was statistically significant when compared with that of LoVo-c2orf68, -HuR cells (P<0.05). *P<0.05.

Figure 5. (A) Compared with the blank group, the number of migrated cells was observably inhibited in the SW480-c2orf68, -HuR, SW480-c2orf68 and SW480-HuR cells. However, the number of SW480-c2orf68, -HuR cells that migrated was observably less than that of SW480-c2orf68 and SW480-HuR cells (P<0.05). (B) The apoptosis rates of SW480-c2orf68, -HuR, SW480-c2orf68 and SW480-HuR cells were significantly increased (P<0.05). (C) Compared with the blank group, cell proliferation was significantly decreased in the SW480-c2orf68, -HuR, SW480-c2orf68 and SW480-HuR cells (P<0.05). Furthermore, the proliferation of SW480-c2orf68, -HuR cells was significantly lesser than that of SW480-c2orf68 and SW480-HuR cells (P<0.05). *P<0.05.
significantly higher than that of LoVo+ c2orf68, HuR, and LoVo cells (Fig. 4A, P<0.05).

Cell apoptosis, cell proliferation and cell migration of SW480- c2orf68, HuR- and SW480-HuR cells. The apoptosis rate of SW480- c2orf68, HuR- cells was significantly higher than that of SW480+ c2orf68, HuR+, SW480- HuR, SW480+ NC and SW480 cells (Fig. 5B, P<0.05). In addition, the apoptosis rate of SW480+ c2orf68 and SW480-HuR cells was significantly higher than that of SW480- c2orf68 and SW480 cells, respectively (Fig. 5B, P<0.05). Compared with the control group, cell proliferation was significantly decreased in the SW480+ c2orf68, HuR+, SW480+ c2orf68 and SW480-HuR cells (Fig. 5C, P<0.05); cell proliferation in the SW480+ c2orf68, HuR+ cells was significantly less than that of the SW480+ c2orf68 and SW480-HuR cells (Fig. 5C, P<0.05). The number of migrated cells of SW480+ c2orf68, HuR+, SW480+ c2orf68, SW480-HuR, SW480- NC and SW480 were 122±16, 234±51, 233±48, 381±42 and 401±24, respectively. Compared with the control group, the number of migrated cells were significantly inhibited in the SW480+ c2orf68, HuR+, SW480+ c2orf68 and SW480-HuR cells (Fig. 5A, P<0.05) cells. However, the number of migrated SW480+ c2orf68, HuR- cells was significantly lower than that of the SW480+ c2orf68 and SW480-HuR cells (Fig. 5A, P<0.05).

mRNA and protein expression of C2orf68, HuR, Bcl-2, Bax, c-Myc, cyclin D and cyclin A in LoVo+ c2orf68, HuR and LoVo- c2orf68 cells. As shown in Fig. 6C, following transfection for 48 h, c2orf68 gene expression was significantly overexpressed in the LoVo+ c2orf68 (P<0.01) and LoVo- c2orf68, HuR cells (P<0.05) when compared with the control group. Similarly, HuR, Bcl-2 and c-Myc were significantly overexpressed in the LoVo+ c2orf68 (P<0.01, P<0.01 and P<0.05, respectively) and LoVo+ c2orf68, HuR cells (all P<0.05). Cyclin D and Cyclin A were significantly overexpressed in the LoVo+ c2orf68 cells (P<0.05), while the upregulation of cyclin D and Cyclin A mRNA expression levels in LoVo+ c2orf68 cells exhibited no significances. The mRNA expression of c2orf68, HuR, Bcl-2, c-Myc and Cyclin A in LoVo+ c2orf68 cells was significantly increased, while cyclin D in LoVo+ c2orf68, HuR cells exhibited no statistical difference with LoVo+ c2orf68 cells. In contrast, the mRNA expression level of Bax was decreased in the LoVo+ c2orf68 (P<0.05) and LoVo+ c2orf68, HuR (P<0.01) cells, when compared to the control. Compared with the LoVo+ c2orf68, HuR cells, the mRNA expression of Bax in the LOVO- c2orf68 cells was significantly decreased (P<0.05).

As shown in Fig. 6A and B, following transfection for 48-72 h, compared to the blank group, the protein expression level of C2ORF68 was significantly increased in the LoVo+ c2orf68, HuR (P<0.05) and LoVo+ c2orf68 (P<0.01) cells. In addition, HuR, BCL-2, C-MYC and Cyclin A protein expression levels were overexpressed in the LoVo+ c2orf68, HuR (NS, P<0.05, P<0.01 and P<0.01, respectively) and LoVo+ c2orf68 cells (P<0.05, P<0.01, P<0.01 and P<0.01, respectively) when compared to the blank group; compared with the LoVo+ c2orf68, HuR cells, the protein expression of C2ORF68, HuR, BCL-2, C-MYC and Cyclin A in LoVo+ c2orf68 cells was increased (P<0.05, P<0.05, P<0.01, P<0.01 and P<0.01, respectively). In contrast, the protein expression level of BAX was decreased in the LoVo+ c2orf68, HuR (P<0.05) and LoVo+ c2orf68 cells (P<0.01). Compared with LoVo+ c2orf68, HuR cells, the protein expression of BAX (P<0.01) in LoVo+ c2orf68 cells was significantly decreased.


**Figure 7.** (A and B) The protein expression levels of C2ORF68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A were significantly decreased. The inhibition rate of C2ORF68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A were significantly higher in the SW480-c2orf68-HuR cells than in SW480-2orf68 and SW480-HuR cells. However, the inhibition rate of CYCLIND was not statistically significant. Furthermore, the mRNA expression level of BAX was significantly increased in the SW480-c2orf68-HuR cells, compared with SW480-2orf68 and SW480-HuR cells. Meanwhile, the protein expression level of BAX was significantly increased in the SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells. (C) In the SW480-2orf68-HuR and SW480-c2orf68-HuR cells, the mRNA expression levels of C2orf68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A were significantly decreased. Furthermore, the inhibition rate of c2orf68, HuR, Bcl-2 and cyclin A in SW480-2orf68-HuR cells was significantly higher than that in the SW480-2orf68 and SW480-HuR cells. The inhibition rate of cyclin D in SW480-2orf68-HuR cells compared with SW480-HuR cells, was statistically significant. However, compared with SW480-2orf68 cells, the inhibition of cyclin D was not statistically significant. Furthermore, the mRNA expression level of BAX was significantly increased in the SW480-2orf68-HuR, SW480-2orf68 and SW480-HuR cells (P<0.01, P<0.01), and the expression of BAX was also increased in the SW480-2orf68-HuR cells when compared with the SW480-2orf68 (P<0.05) and SW480-HuR (P<0.05) cells. *P<0.05, **P<0.01.

**mRNA and protein expression of c2orf68, HuR, Bcl-2, Bax, c-Myc, cyclin D and cyclin A in SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells.** As shown in Fig. 7C, in SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells, the mRNA expression level of c2orf68, HuR, Bcl-2, c-Myc, cyclin D and cyclin A significantly decreased (P<0.05). Furthermore, the inhibition rate of c2orf68, HuR, Bcl-2, c-Myc and cyclin A in SW480-c2orf68-HuR cells was significantly higher than that in SW480-2orf68 and SW480-HuR cells (P<0.05). The inhibition rate of cyclin D in SW480-c2orf68-HuR cells compared with SW480-HuR cells, was statistically significant (P<0.05). However, compared with SW480-2orf68 cells, the inhibition of cyclin D was not statistically significant. Meanwhile, the increase in Bax mRNA expression was significantly higher in SW480-c2orf68-HuR cells than in SW480-2orf68 and SW480-HuR cells (P<0.05). Furthermore, the mRNA expression level of Bax was increased in SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells.

As shown in Fig. 7A and B, following transfection for 48-72 h, compared to the blank group, the protein expression level of C2ORF68 was significantly decreased in the SW480-c2orf68-HuR (P<0.01), SW480-2orf68 (P<0.01) and SW480-HuR cells (P<0.01). Similarly, the protein expression levels of HuR, BCL-2, C-MYC, Cyclin D and Cyclin A were also decreased in the SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells. Furthermore, the inhibition rate of C2ORF68 (P<0.01, P<0.01), HuR (P<0.05, P<0.05), C-MYC (P<0.05, P<0.01) and Cyclin A (P<0.05, P<0.01) was significantly higher in the SW480-c2orf68-HuR cells than that in the SW480-2orf68 and SW480-HuR cells. However, the inhibition rate of Cyclin D in SW480-c2orf68-HuR cells, compared with SW480-2orf68 and SW480-HuR cells, was not statistically significant. Meanwhile, the protein expression level of BAX was significantly increased in the SW480-c2orf68-HuR, SW480-2orf68 and SW480-HuR cells when compared with the blank group. Compared with the SW480-2orf68 and SW480-HuR cells, the protein expression level of BAX was significantly higher in the SW480-c2orf68-HuR cells (P<0.01, P<0.01).

**Discussion**

In the present study, it was shown that the expression level of C2ORF68 was significantly upregulated in rectal cancer tissues compared with its adjacent normal tissues by IHC. This indicates that c2orf68 may be involved in the occurrence of rectal cancer. In addition, upregulated expression of C2ORF68 was significantly correlated with a variety of important clinicopathological parameters, including pathological grade and lymph node metastasis. It was suggested that C2ORF68 may play a role in the development and metastasis of CRC. As a result, a statistical significance was found between the expression of C2ORF68 and pathological grade. This indicates that the expression level of C2ORF68 may be associated with the malignant potential of cancer. That is, the higher the expression level of C2ORF68, the higher is the degree of malignancy of...
rectal carcinoma. These present results suggest that the c2orf68 gene is associated with the occurrence and development of rectal cancer and may be a potential carcinogenic factor in rectal cancer. However, the detailed mechanism for c2orf68 upregulation in rectal cancer remains to be clarified.

Through bioinformatic analyses, we discovered that there are 12 proteins which interact with C2orf68, including KLHL15, GMNN, SMG6, GNE, DDX22, PIR, ELAVL1 (HuR), NAGK, XPO1, HSPA9, JOSD2 and GUH1 (BioGrid database). At the same time, by our experiments, including IF and Co-IP, we verified that C2orf68 co-localized with HuR in SW480 and LoVo cell lines, and C2orf68 could interact with HuR. HuR, a member of the Hu/ELAV family, is predominantly located in the nucleus and translocates to the cytoplasm when cells are stimulated by endogenous factors or external stimuli (21,22). HuR (ELAV1) is an RNA binding protein, which has been shown to regulate the expression of multiple genes by different post-transcriptional mechanisms, such as mRNA decay and protein translation (23). HuR modulates posttranscriptional processing of target premRNAs or mRNA stabilization and translation through interaction with AU-rich elements (ARE) within 3'-untranslated regions (UTRs) of the target mRNAs to transformation (24). Furthermore, it has been shown that HuR stabilizes mRNAs that encode p53 and WEE1 (25), activates ATF2 (26), Jun D (27) and XIAP (28) and enhances the translation of mRNAs that encode c-Myc (29), ICH-1 (30) and IL-1β (31). Many of these transcripts are reported to participate in certain key cellular processes including cell proliferation, cell apoptosis, angiogenesis, immune response and metastasis. HuR is also increased in malignant cells when compared with corresponding normal cells, and it has been found to be associated with adverse clinicopathological factors in several different cancer types, such as gastric, gallbladder breast, urothelial and non-small cell lung cancer (32).

Our study revealed that cell apoptosis increased, cell proliferation and cell migration decreased when c2orf68 was inhibited in SW480 cells. These results are consistent with our previous study (16). In addition, we also demonstrated that cell apoptosis increased while cell proliferation and cell migration decreased in SW480 +HuR and SW480 -c2orf68 cells. This indicates that both c2orf68 and HuR can regulate cell apoptosis and proliferation in CRC cells. The cell apoptosis rate, cell proliferation and cell migration in SW480 -c2orf68, +HuR cells which has more significant results than that in SW480 +HuR cells revealed that c2orf68 and HuR may have a synergistic effect in regulating cell apoptosis, cell proliferation and cell migration. This study differed from our previous study (16), which focused on the PI3K/Akt/mTOR signaling pathway and its downstream molecules, such as Akt, PI3K, Bcl-2, c-Myc, cyclin D1 and bax when c2orf68 was inhibited. A recent study showed that the HuR expression level is closely related to AKT phosphorylation and PI3K/AKT/NF-kB signaling can notably elevate HuR gene transcription (18). This study focused on the relationship between C2orf68 and HuR and the downstream molecules of HuR, such as Bcl-2, Bax, c-Myc, cyclin D and cyclin A in SW480 +c2orf68, +HuR, SW480 +c2orf68 and SW480 -HuR cells. According to our results, Bcl-2, c-Myc, cyclin D and Cyclin A decreased, and Bax increased in the SW480 +c2orf68, +HuR, SW480 +c2orf68 and SW480 -HuR cells. In addition, Bcl-2, c-Myc, cyclin D and cyclin A was significantly decreased and Bax was significantly increased in SW480 -c2orf68, +HuR cells. This shows that c2orf68 and HuR may co-regulate Bcl-2, c-Myc, cyclin D, cyclin A and Bax, resulting in the cell apoptosis and cell proliferation CRC cells.

In the present study, the mRNA and protein expression of HuR was downregulated when c2orf68 was inhibited in SW480 cells, and the mRNA and protein expression of HuR was upregulated when c2orf68 was overexpressed in LoVo cells. Furthermore, when HuR was inhibited, the mRNA and protein expression levels of c2orf68 were also decreased. That is, HuR and c2orf68 had a synergistic effect.

The present study revealed that cell apoptosis decreased while cell proliferation and cell migration were increased in LoVo +c2orf68 cells. These results are consistent with our previous study (17), and it was confirmed that c2orf68 can regulate cell apoptosis and proliferation. In addition, it was also revealed that cell apoptosis was decreased when cell proliferation and cell migration were increased in LoVo +c2orf68, +HuR cells. However, cell apoptosis was significantly lower and cell proliferation and cell migration were significantly higher in LoVo +c2orf68 cells, compared with LoVo +c2orf68, -HuR cells. All these results suggest again that c2orf68 and HuR may have a synergistic effect in promoting cell proliferation and migration, and in inhibiting cell apoptosis in the role of CRC. Unlike our previous study, which focused on the Wnt signaling pathway and its molecules such as β-catenin, survivin, cyclin D1, c-Myc and GSK-3β (17), the present study focused on the relationship between C2orf68 and HuR and the downstream molecules of HuR such as Bcl-2, Bax, c-Myc, cyclin D and cyclin A. In LoVo +c2orf68 and LoVo +c2orf68, +HuR cells, Bcl-2, c-Myc, cyclin D and cyclin A increased, while Bax decreased. In addition, Bcl-2, c-Myc, cyclin D and cyclin A were significantly upregulated and Bax was significantly decreased in LoVo +c2orf68 cells, compared with LoVo +c2orf68, -HuR cells. Previously in this manuscript, we described that Bcl-2, c-Myc, cyclin D and cyclin A were decreased, while Bax was increased in the SW480 +c2orf68, +HuR, SW480 +c2orf68 and SW480 -HuR cells. In addition, Bcl-2, c-Myc, cyclin D and cyclin A were significantly decreased and Bax was significantly increased in the SW480 -c2orf68, -HuR cells, compared with the SW480 +c2orf68, +HuR cells. It is known that all these genes are involved in key cellular processes such as cell proliferation and cell cycle. The expression of cyclin D1 is increased in many tumors and it promotes cell proliferation by regulating cell cycle progression through the G1/S restriction point (33). The transcription factor c-Myc, which is a leucine zipper protein regulating the expression of 10-15% of human genes, plays an important role in cell proliferation, differentiation, growth and survival. Its overexpression is associated with cancer occurrence and development (34). BCL-2, an anti-apoptosis protein, belongs to the Bcl-2 family, and was firstly discovered in B cell malignancies and regulates the intrinsic mitochondrial apoptosis pathway (35). Activated Bax then oligomerizes at the mitochondria to induce outer mitochondrial membrane (OMM) permeabilization and releases into the cytosol apoptotic factors which promote caspase activation and subsequent apoptosis execution (36). Cyclins are fundamental regulators of the cell cycle, playing an important role in tumorigenesis, Cyclin A is required for cells to progress through the S phase (37). As a result, it is believed that c2orf68...
and HuR may have a synergistic effect and may co-regulate cell proliferation and apoptosis by regulating the downstream molecules of HuR, such as upregulating Bcl-2, c-Myc, cyclin D and cyclin A gene expression and downregulating Bax gene expression, resulting in the development of colorectal cancer. Furthermore, it may be associated with lymph node metastasis of colorectal cancer. The carcigenesis of c2orf68 may be related to the promotion of cell proliferation and inhibition of cell apoptosis. C2orf68 may have a synergistic effect with HuR, and the possible mechanism of c2orf68 and HuR involves the co-promotion of cell proliferation and migration, and the co-inhibition of cell apoptosis. However, the exact mechanism remains mysterious. C2orf68 and HuR may co-regulate cell proliferation and apoptosis by upregulating Bcl-2, c-Myc, cyclin D and cyclin A gene expression and downregulating Bax gene expression, resulting in the development of colorectal cancer. Our previous study indicated that C2ORF68 can regulate cancer cell proliferation and apoptosis through PI3K/AKT/mTOR signaling (16). At the same time, a recent study (18) showed that the HuR expression level is closely related to AKT phosphorylation and cytoplasmic abundance of HuR in human cancer may be associate with oncogenic activation of AKT signaling. According to the above conclusions, we may hypothesize that C2ORF68, HuR and AKT signaling could make up a mutually reinforcing loop, regulating cancer cell proliferation and apoptosis. However, the specific mechanism warrants further research.

Acknowledgements

I need to express my sincere gratitude to all authors for the assistance with this article, especially to Professor Yao Chen, whose constant encouragement directed me through all stages of the writing of this manuscript. Without her help, I could not have gotten to this point. Furthermore, I also extend my heartfelt gratitude to the other authors, and thanks for their efforts.

Funding

The present study was supported by Chengdu Department of Science and Technology (2018-YF05-00-038-SN), Sichuan Province, China.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

LS, KS, TJ and YC conceived and designed the study. ZL and KH performed the experiments. ZL and KH wrote the paper. LS, KS, TJ and YC reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Medical Research Ethics Committee of Sichuan University approved the sample acquisition (Chengdu, China), and a written informed consent was also obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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