HSPA4 knockdown retarded progression and development of colorectal cancer

Mingliang Zhang
First Affiliated Hospital of Sun Yat-sen University

Weigang Dai
First Affiliated Hospital of Sun Yat-Sen University

Zhanyu Li
First Affiliated Hospital of Sun Yat-Sen University

Liang Tang
First Affiliated Hospital of Sun Yat-Sen University

Jianhui Chen
First Affiliated Hospital of Sun Yat-Sen University

Chuangqi Chen (✉ drcqc2001@outlook.com)
Sun Yat-sen University First Affiliated Hospital https://orcid.org/0000-0001-9138-3868

Primary research

Keywords: CRC, HSPA4, proliferation, apoptosis, migration

Posted Date: August 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-54159/v2

License: ☺️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of cancer death. The heat shock 70kDa protein 4 (HSPA4) participate in progression and development of cancers. However, the cellular functions, potential molecular mechanisms of HSPA4 in CRC are still largely unknown. **Methods:** In this study, qRT-PCR and Western Blot were used to identify the constructed HSPA4 knockdown cell lines, which was further used to construct mouse xenotransplantation models. Effects of HSPA4 knockdown on cell proliferation, apoptotic, cell cycle and migration of CRC were examined using Celigo cell counting assay, Flow cytometry, wound healing assay and Transwell assay, respectively. In addition, Human Apoptosis Antibody Array was performed to explore downstream molecular mechanism of HSPA4 in CRC cells.

**Results:** HSPA4 was overexpressed in CRC, which was positively associated with lymphatic metastasis (N value), number of Lymph node. In addition, high expression of HSPA4 predicted poor prognosis of patients with CRC. Furthermore, HSPA4 knockdown inhibit proliferation, migration, promote apoptosis, and arrest cell cycle of CRC cells in vitro. Moreover, in vivo results supported HSPA4 knockdown inhibit tumor growth. Additionally, the induction of apoptosis of CRC cells by HSPA4 knockdown required the participation of a series of apoptosis-related proteins. The downregulation of HSPA4 promoted the progression of CRC cells, which resulted in alterations of PI3K/Akt, CCND1 and CDK6 in downstream signaling pathways.

**Conclusions:** In sum, the downregulation of HSPA4 promoted CRC and may be a potential target for molecular therapy.

Introduction

Nowadays, colorectal cancer (CRC) is identified as the fourth leading cause of cancer death worldwide and with high incidence [1]. CRC prevalence is expected to increase by 60%, which attribute over than 1.1 million of death and 2.2 million new cases by the year 2035 [2]. On the other hand, mutations in specific genes may contribute to the onset of CRC, as happens in other types of cancer. Those mutations can appear in oncogenes, tumor suppressor genes and genes related to DNA repair mechanisms [3]. Current treatment for CRC involves a variety of approaches, including surgery and radiotherapy, as well as the use of chemotherapeutic drugs such as FOLFOX and FOLFIRI [4-6]. Additionally, monoclonal antibodies or proteins against vascular endothelial growth factor (VEGF) and epidermal growth receptor (EGFR) combined with traditional chemotherapy have been applied to improve the outcome of CRC [7]. Despite the advanced therapies, CRC patients still have poor survival rate and high recurrence outcome [8]. Consequently, there is an urgent need to thoroughly understand the molecular mechanisms of CRC, identify key molecular targets, and lay the foundation for the development of new and more effective treatments.
Heat shock proteins (HSPs) are ubiquitous molecules within cells that acts as a molecular chaperone under stress conditions, including carcinogenesis [9]. HSPs part are classified based on its molecular size, and heat shock 70kDa protein 4 (HSPA4) is a member of the HSP110 family [10]. Previous studies have found a highly abundant set of HSPs on the surface of tumor cells, including HSPA4, HSP60, HSP70, HSP90, and HSP27[9, 11]. Moreover, frameshift mutations of HSPA4 in gastric cancer and CRC was proved by Jo et al., [12]. Emerging evidence suggested that HSPA4 participate in progression and development of cancers. For example, Wang et al., indicated that HSPA4 may be important for nasopharyngeal carcinoma (NPC) metastasis [13]. Ma et al., found a significant correlation between HSPA4 expression and overall survival in hepatocellular carcinoma (HCC) patients [14]. Gu et al., proposed that tumor-educated B cells selectively promote breast cancer lymph node metastasis by HSPA4-targeting IgG [15]. Interestingly, Han et al., inferred that inhibition of LPS-induced cardiomyocyte apoptosis and mitochondrial damage by suppressing post-transcriptional regulation of HSPA4 by miR-1-5p [16]. Although many HSPA4 have been reported to be involved in tumor biological processes, the cellular functions, potential molecular mechanisms of HSPA4 in CRC are still largely unknown.

In the present study, we have made an effort to elucidate the relationship of HSPA4 in CRC. To this end, we identified that HSPA4 is upregulated in CRC and associated with poor prognosis. More importantly, in vitro experiments proved that knockdown HSPA4 may inhibit cell proliferation, migration, promote cell apoptosis, and arrest cell cycle. Meanwhile, in vivo results also supported HSPA4 knockdown inhibit tumor growth. In this context, it is plausible to suggested that HSPA4 may contribute to development and progression of CRC. Therefore, these findings suggested that HSPA4 may be a potential therapeutic target in CRC.

Materials And Methods

CRC tissue samples and cell lines

101 cases of formalin-fixed paraffin-embedded human CRC and their paired normal tissue sections were purchased from Shanghai Outdo Biotech Company. None of the patients received radiotherapy or chemotherapy prior to biopsy sampling. This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University, and written informed consent was obtained from each participant.

The human CRC cell line HCT116 and RKO were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of the cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) in a 5% CO₂ atmosphere. Notably, all CRC cell lines were tested for mycoplasma contamination.

Immunohistochemical (IHC) Staining

Briefly, the specimens were deparaffinized and blocked with citric acid antigen. Subsequently, antibody HSPA4 (1: 200, abcam, USA, # ab185962) was added at 4°C overnight and elution with PBS, next
antibody IgG (1: 400, abcam, USA, # ab6721) was added. Tissue slices were stained with DAB and hematoxylin. All tissues in the chip were observed with microscopic and all slides were viewed with ImageScope and CaseViewer. IHC scores were determined by staining percentage scores (classified as: 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), 4 (75%-100%)) and staining intensity scores (scored as 0: signalless color, 1: brown, 2: light yellow, 3: dark brown). Pathological examination of tumor specimens was carried out by two independent pathologists.

Target gene RNA interferes with the preparation of lentiviral vector

Short hairpin RNAs (shRNA) of human HSPA4 and related control sequence were designed by Shanghai biosciences Co., Ltd. for knockdown experiments. The relevant sequence information was shown in the Table below. Afterwards, target sequences were inserted into BR-V-108 vector (Shanghai biosciences Co. Ltd., Shanghai, China) using the T4 DNA ligase enzyme (NEB). Plasmids were extracted by EndoFree Maxi Plasmid Kit (Tiangen) and qualified plasmid was packaged with 293T cells. HCT116 and RKO cells at a density of 2×10^5 cells/mL were seeded in a 6-well plate. 24 h later, cells were transfected with 100 μL lentiviral vectors (1×10^7 TU/mL) additive with ENI.S and polybrene (10 µg/mL, Sigma-Aldrich). After cultured for 72 h, the fluorescence was observed by microscope and then transfection efficiency detected via qRT-PCR and Western Blot.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human-HSPA4 (RNAi-1)</td>
<td>AAAGTCAAGGTTCCGAGTAAAT</td>
</tr>
<tr>
<td>Human-HSPA4 (RNAi-2)</td>
<td>GGTGACATATATGGAGGAAGA</td>
</tr>
<tr>
<td>Human-HSPA4 (RNAi-3)</td>
<td>AAGCAATGGAGTGGATGAATA</td>
</tr>
</tbody>
</table>

qRT-PCR

Total RNA was extracted from cell lines HCT116 and RKO with TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. RNA (2.0 μg) was transcribed into cDNA with M-MLV RT kit (Promega). GAPDH was used as the internal controls for the quantification of HSPA4 (primer sequences were detailed in Table). The qRT-PCR was carried out on the Roche Light Cycler® 96 real-time PCR platform, and expression of HSPA4 was quantified using the 2^−ΔΔCT method.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Upstream Sequence (5’-3’)</th>
<th>Downstream Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPA4</td>
<td>GCAGACACCACGCAAAAAATAAGG</td>
<td>TCGATTGGCAGGTCCACAGT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGACTTCAACACAGCACCCCA</td>
<td>CACCTGTGTGCTGTAGCCAAA</td>
</tr>
</tbody>
</table>

Western Blot

HCT116 and RKO cells transfected with shCtrl and shHSPA4 were fully lysed in ice-cold RIPA buffer (Millipore). The protein concentration detection was performed by BCA Protein Assay Kit (#23225,
HyClone-Pierce). 20 μg protein from each group was separated by 10% SDS-PAGE, transferred onto PVDF membranes, and analyzed with required antibodies (antibody information was detailed in Table). The blots were visualized by Amersham ECL plusTM Western Blotting system and the density of the protein band was analyzed.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Protein Size (KDa)</th>
<th>Diluted Multiples</th>
<th>Antibody Source</th>
<th>Company</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPA4</td>
<td>94</td>
<td>1:2000</td>
<td>Rabbit</td>
<td>abcam</td>
<td>ab185962</td>
</tr>
<tr>
<td>Akt</td>
<td>60</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>CST</td>
<td>4685</td>
</tr>
<tr>
<td>p-Akt</td>
<td>60</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Bioss</td>
<td>BS-5193R</td>
</tr>
<tr>
<td>CCND1</td>
<td>36</td>
<td>1:2000</td>
<td>Rabbit</td>
<td>CST</td>
<td>2978</td>
</tr>
<tr>
<td>CDK6</td>
<td>37</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>abcam</td>
<td>ab151247</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>110</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>abcam</td>
<td>ab40776</td>
</tr>
<tr>
<td>GAPDH</td>
<td>37</td>
<td>1:3000</td>
<td>Rabbit</td>
<td>Bioworld</td>
<td>AP0063</td>
</tr>
</tbody>
</table>

**Celigo cell counting assay**

HCT116 and RKO cells transfected with shCtrl and shHSPA4 were seeded into a 96-well plate (500 cells/well) and cultured until cell clones formed. After that, cell clones were fixed with 4% paraformaldehyde and then stained with Giemsa. The visible colonies were photographed and counted for 5 consecutive days.

**Flow cytometry**

HCT116 and RKO cells transfected with shCtrl and shHSPA4 were inoculated in a 96-well plate until cell density reached 85%. Cells were harvested, centrifuged (1200 × g), and resuspended. Apoptosis analyses and cell cycle distribution was detected Guava easyCyte HT FACS Calibur (Millipore).

In terms of cell apoptosis, 10 μL Annexin V-APC (eBioscience) was added and incubated at room temperature without light for 10 min. Cell apoptotic ratio was calculated based on the following formula: (number of positive cells/number of all counted cells) × 100%.

For cell cycle, cells were stained by PI staining solution (BD Biosciences). The ratio of cells in the G1, S and G2 stage of the HSPA4 knockdown group and the control group were detected and analyzed by flow cytometry.

**Wound healing assay**

Cell migration was measured by a scratch wound healing assay. HCT116 and RKO cells transfected with shCtrl and shHSPA4 were plated into a 96-well dish (5×10^4 cells/well) for culturing until the confluence
reached 90%. Subsequently, cell scratches across the cell layer were made by a 96-wounding replicator (VP scientific). After cell layers were gently washed, photographs were taken by a fluorescence microscope at 24 h and 48 h post scratching and the migration rates were calculated.

**Transwell Assay**

8×10^4 HCT116 and RKO cells transfected with shCtrl and shHSPA4 were seeded in a 24-well Transwell cell culture plate, respectively, in the upper chamber. The lower chamber was filled with 600 μL medium and supplemented with 30% FBS. After 24 h culturing, the non-metastatic cells were washed away gently and metastatic cells were stained with 400 μL Giemsa for 5 min at room temperature. Cells were observed via a microscope (200 × magnification) and the migration ability of cells was analyzed.

**Animal Xenograft Model**

Animal research experiments were approved by the Ethics committee of the First Affiliated Hospital of Sun Yat-sen University and conducted in accordance with guidelines and protocols for animal care and protection. BALB/c nude mice (female, 4 weeks old) obtained from Shanghai Lingchang Laboratory Animal Technology Co., Ltd. 4 × 10^6 RKO cells transfected with shCtrl and shHSPA4 were subcutaneously injected into each mouse under the armpit of the right forearm (n=10 per group). The tumor size and weight were monitored 2 times per week, and tumor volume =π/6× L × W, where L represents the long diameter and W represents the short diameter. After 26 days of injection, all mice were anesthetized by intraperitoneal injection of 0.7% Pentobarbital Sodium (10 μL/g), and anesthetized mice were placed under the Berthold Technologies living imaging system and in vivo bioluminescence images was collected. Then anesthetized mice were sacrificed and the tumor tissues were harvested.

**Ki67 Staining**

Mice tumor tissues were fixed in 10% formalin and then were paraffin-embedded. 5 μm slides were cut and immersed in xylene and ethanol. Tissue slides were blocked with 3% PBS-H₂O₂ and were incubated with anti-Ki67 and HRP goat anti-rabbit IgG. Slides were stained by Hematoxylin (Baso, # BA4041) and Eosin (Baso, # BA4022). Stained slides were examined at 200× and 400× objective lens microscopic.

**Human Apoptosis Antibody Array**

For signal pathway gene detecting, Human Apoptosis Antibody Array (abcam, # ab134001) were applied following the manufacturer’s instructions. Briefly, RKO cells transfected with shCtrl and shHSPA4 were lysed in cold RIPA buffer (Millipore) and protein concentration was detected by BCA Protein Assay Kit (HyClone-Pierce). Proteins were incubated with blocked array antibody membrane overnight at 4°C. After washing, 1:100 Detection Antibody Cocktail was added incubating for 1 h, followed by incubated with HRP linked streptavidin conjugate for 1 h. All spots were visualized by enhanced ECL and the signal densities were analyzed with ImageJ software (National Institute of Health).

**Statistical Analysis**
All experiments were performed in triplicate and data were shown as mean ± SDs. Statistical analyses and graphs were performed by GraphPad Prism 8.01 (Graphpad Software) and P value < 0.05 as statistically significant. The significance difference between groups were determined using the two-tailed Student’s t test or One-way ANOVA analysis. Mann-Whitney U analysis and Spearman rank correlation analysis were used while explaining the relationships between HSPA4 expression and tumor characteristics in patients with CRC.

Results

Upregulated HSPA4 is associated with poor prognosis in CRC patients

To evaluate the role of HSPA4 in CRC, we first examined whether the expression level of HSPA4 was abnormal in CRC. IHC results presented that the expression of HSPA4 was higher in CRC tissues than that in normal tissues (P < 0.001) (Fig. 1A) (Tab. 1). Subsequently, according to Mann-Whitney U analysis, there was a significant correlation between HSPA4 expression and pathological data, such as lymphatic metastasis (N value) and number of Lymph node positive (Tab. 2). Interestingly, Spearman grade correlation analysis further confirmed this conclusion (Tab. 3). In other words, the tumor malignancy of CRC patients deepened the upregulation of HSPA4 expression. More importantly, Kaplan-Meier survival analysis revealed that HSPA4 expression levels were significantly associated with the overall survival of CRC patients (Fig. 1B). In other hand, the mRNA expression of HSPA4 in CRC cell lines (HCT116, RKO, DLD-1, Caco2, SW480) was upregulated detected by qRT-qPCR (Fig. 1C). Comprehensive analysis showed that HSPA4 may be related to the development and prognosis of CRC.

Construction of HSPA4 knockdown cells models

To assess the function of HSPA4 in CRC, loss-of-function cells models was designed by silencing HSPA4 in HCT116 and RKO cells. First, results of qRT-qPCR showed that shHSPA4 (RNAi-3) group presented the best knockdown efficiency (Fig.1D). Therefore, shHSPA4 (RNAi-3) group was used for subsequent experiments. Fluorescence imaging was made 72 h after transfection of HCT116 and RKO with shHSPA4 or shCtrl, and the results showed that the transfection efficiency reached over 80% (Fig. 2A). Moreover, data of qRT-PCR displayed that knockdown efficiencies of HSPA4 in HCT116 and RKO were 96.1% (P < 0.01) and 94.94% (P < 0.01), compared with the shCtrl groups, respectively (Fig. 2B). Afterwards, Western Blot results also verified that HSPA4 knockdown successful (Fig. 2C). All in all, HSPA4 knockdown cell models were successfully constructed.

Knockdown of HSPA4 inhibits CRC cell proliferation in vitro

Subsequently, Celigo cell counting assay was performed to detected cell proliferation.

The effects of HSPA4 knockdown on the development of HCT116 and RKO cells were observed by cell counts, which indicated that compared with shCtrl group, the cell proliferation inhibition of shHSPA4
group was obvious (P < 0.001) (Fig. 3A). This experimental result suggested that HSPA4 may play a vital role in the cell proliferation of CRC.

**Knockdown of HSPA4 promotes CRC cell apoptosis and arrests cell cycle in vitro**

To further investigate the role of HSPA4 in the development of CRC, flow cytometry was applied to access the apoptotic ratio and cell cycle among the cells transfected with shHSPA4 or shCtrl. On the one hand, compared with shCtrl group, shHSPA4 group apoptosis rates increased by almost 2 folds of HCT116 cells, while that in RKO cells increased by 3.32 folds (P < 0.001) (Fig. 3B). On the other hand, the G2 phase cells numbers of HCT116 and RKO were increased in shHSPA4 group compared to the shCtrl group (P < 0.01) (Fig. 3C). Combined with above results, we estimated that HSPA4 knockdown promotes cell apoptosis and arrests cell cycle progression in CRC.

**Knockdown of HSPA4 inhibits CRC cell migration in vitro**

Furthermore, effects of HSPA4 on migration of CRC cell were detected through wound healing assay and Transwell assay. During 48 h of HCT116 cells, migration rate of shHSPA4 group was reduced by 39% compared to shCtrl group (P < 0.001), while there were no significant change RKO cells (Fig. 4A). Additionally, migration fold change of HCT116 and RKO was evaluated by Transwell assay, results expressed that migration rate decreased by about 60% and 90%, respectively in shHSPA4 group compared to the shCtrl group (P < 0.01) (Fig. 4B). Accordingly, HSPA4 knockdown has certain ability to inhibit CRC cell migration.

**Knockdown of HSPA4 in CRC cells impairs tumorigenesis in vivo**

The above results validated that knockdown of HSPA4 could inhibit cell proliferation, migration and promote apoptosis in vitro. This experiments further explored whether knockdown of HSPA4 was consistent in vivo. All procedures involving mice and experimental protocols were approved by the Institutional Animal Care and Use Committees of the First Affiliated Hospital of Sun Yat-sen University. RKO cells with or without HSPA4 knockdown were subcutaneously injected into nude mice to establish mouse xenotransplantation model. Results were shown in Fig. 5A-5C, indicated that volume and weight of tumors in the shHSPA4 group were significantly smaller and lighter than those in the shCtrl group (P < 0.01). In particular, bioluminescence imaging presented that tumor growth in shHSPA4 group was slower than that in shCtrl group (P < 0.05) (Fig. 5D, 5E). In addition, images of Ki67 staining (magnification: 200 × and 400×) showed that the proliferation index of tumor tissues in shHSPA4 group was significantly lower than that in the shCtrl group (Fig. 5F). Clearly, the in vivo and in vitro experimental conclusions were consistent, suggesting that HSPA4 knockdown attenuated tumorigenicity in CRC.

**Exploration of downstream molecular mechanism of HSPA4 in CRC cell**

To validate the potential mechanism of the regulation ability of HSPA4 knockdown in CRC, Human Apoptosis Antibody Array was performed to analyze the differential expression of 43 proteins in RKO cells between shHSPA4 and shCtrl groups. The results were presented in Fig. 6A, 6B, protein expression of
Caspase3, HSP60, IGFBP-6 and SMAC were significantly upregulated in RKO cells with HSPA4 silencing. Conversely, the expression levels of Bcl-2, Bcl-w, IGF-I, IGF-II and IGF-1sR was downregulated by HSPA4 knockdown (P < 0.05). Furthermore, the expression of HSPA4 downstream signaling proteins with the aid of Western Blot was also detected.

Protein bands showed that expression of p-Akt, CCND1, CDK6, PIK3CA was downregulated, while Akt expression was not significantly changed in shHSPA4 group compared with shCtrl (Fig. 6C). Therefore, HSPA4 was involved in the progression of CRC cell function, which not only required a series of apoptotic related proteins to participate, but also caused changes of downstream signaling pathways.

Discussion

HSPA4 have been reported to participate in diverse biological processes, including progression and development of cancers. In the present study, we found that the expression of HSPA4 is highly expressed in CRC, which can predict poor prognosis. In other hand, knockdown of HSPA4 inhibited CRC cell proliferation, migration, promoted apoptosis, and arrested cell cycle in vitro. Meanwhile, in vivo experiments also supported HSPA4 knockdown inhibit tumor growth.

In addition, HSPA4 was involved in the development of CRC cell function, which required a series of apoptotic related proteins to participate. Pro-apoptosis protein expression of was significantly upregulated in CRC cells with HSPA4 silencing, such as Caspase3, HSP60, IGFBP-6 and SMAC. Conversely, the expression levels of Bcl-2, Bcl-w, IGF-I, IGF-II and IGF-1sR was downregulated by HSPA4 knockdown. It is well known that escape from apoptosis is the basis of cancer pathogenesis, and apoptosis drives cancer cells proliferate and metastasize [17]. Apoptosis involves a series of biochemical events, which are mediated by a variety of cellular signals [18]. On the one hand, Caspase3 is a major executioner caspase, active Caspase3 degrades multiple cellular proteins and is responsible for cell morphology changes and DNA fragmentation during apoptosis [19, 20]. On the other hand, HSP60 released from mitochondria to cytosol upon death stimuli might exert a pro-death function, either through stabilizing Bax, enhancing Caspase3 activation, or increasing protein ubiquitination [21, 22]. Moreover, Qiu et al., pointed that IGFBP-6 could inhibit invasion and migration of CRC cells possibly via promoting apoptosis activity and arresting cell cycle [23]. Interesting, multiple preclinical studies have documented the ability of SMAC to either directly induce cell death of cancer cells or trigger cell death [24]. Besides, Bcl-2 family has a delicate balancing effect on apoptosis and tumorigenesis [25]. Gu et al., suggested that the induction of apoptosis in CRC cells was a molecular mechanism through the effects on Bcl-2, Bax and Caspase3 [26]. IGFs include IGF-I, IGF-II, relevant receptors IGF-IR and IGF-IIR, and IGF-binding proteins (IGFBPs), all of which exert crucial roles in anti-apoptosis and facilitating cell proliferation [27, 28]. Han et al., illustrated that the occurrence of CRC may be associated with elevated expression levels of IGFs-related proteins [29]. Accordingly, HSPA4 was involved in cell apoptosis of CRC, which subject to a series of apoptotic related proteins to participate.
HSPA4 knockdown participated in the development of CRC cell function, meanwhile triggering changes in downstream signaling protein, such as expression of p-Akt, CCND1, CDK6, PIK3CA was downregulated. Studies have shown that the PI3K/Akt/mTOR pathway includes mutations in PI3K and the activation of Akt that regulate cell growth and survival [30]. Slattery et al., proposed that PIK3CA, a human gene encoding PI3K, also was a vital factor related to post-diagnosis of CRC, suggesting that the genetic variation of PI3K/Akt pathway affects survival [31]. Notably, inhibition of this pathway may be a very effective measure in cancer treatment [32]. Additionally, Bali et al., reported that CCND1 is essential in the pathogenesis and metastasis of CRC and is an important index to judge the prognosis of CRC [33]. Zhang et al., affirmed that targeted therapy with combined use of CDK4/6 inhibitors can provide a promising treatment for CRC [34]. Comprehensive analysis suggested that HSPA4 knockdown inhibited CRC cellular function by inhibiting downstream signaling pathways.

Conclusions

In summary, it was plausible to suggested that HSPA4 may contribute to development and progression of CRC. Therefore, these studies affirmed that HSPA4 may be a potential therapeutic target in CRC.

Declarations

Ethics approval and consent to participate

Animal research experiments were approved by the Ethics committee of the First Affiliated Hospital of Sun Yat-sen University.

This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Funding

Guangdong Science and Technology Plan Project, No. 2013B021800131, Guangzhou health and medical collaborative innovation major projects, No. 201604020003.
Authors’ contributions

Chuangqi Chen designed this program. Mingliang Zhang, Weigang Dai and Zhanyu Li completed experiments on cell function. Liang Tang and Jianhui Chen performed animal experiments. Weigang Dai conducted the data collection and analysis. Mingliang Zhang produced the manuscript which was checked and revised by Chuangqi Chen. All the authors have confirmed the submission of this manuscript.

Acknowledgements

Not applicable.

References

11. Lim SO, Park SG, Yoo JH, Park YM, Kim HJ, Jang KT, et al. Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and


**Tables**

Table 1. Expression patterns in colorectal cancer tissues and para-carcinoma tissues revealed in immunohistochemistry analysis

<table>
<thead>
<tr>
<th>HSPA4 expression</th>
<th>Tumor tissue</th>
<th>Para-carcinoma tissue</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Percentage</td>
<td>Cases</td>
</tr>
<tr>
<td>Low</td>
<td>42</td>
<td>48.3%</td>
<td>62</td>
</tr>
<tr>
<td>High</td>
<td>45</td>
<td>51.7%</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 2. Relationship between HSPA4 expression and tumor characteristics in patients with colorectal cancer
<table>
<thead>
<tr>
<th>Features</th>
<th>No. of patients</th>
<th>HSPA4 expression</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>All patients</td>
<td>87</td>
<td>42</td>
<td>45</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 70</td>
<td>43</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>≥ 70</td>
<td>44</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>Female</td>
<td>43</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5cm</td>
<td>38</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>≥ 5cm</td>
<td>48</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>72</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>III</td>
<td>13</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>T Infiltrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>T3</td>
<td>64</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>T4</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>lymphatic metastasis (N)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>51</td>
<td>30</td>
<td>21</td>
</tr>
<tr>
<td>N1</td>
<td>26</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>N2</td>
<td>10</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Lymph node positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>= 0</td>
<td>49</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>&gt; 0</td>
<td>36</td>
<td>12</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 3. Relationship between HSPA4 expression and tumor characteristics in patients with colorectal cancer
HSPA4 lymphatic metastasis (N) Spearman correlation 0.232
Significance (double-tail) 0.031

N 87

Lymph node positive Spearman correlation 0.256
Significance (double-tail) 0.018

N 85

Figures

Figure 1

HSPA4 is highly expressed in CRC. (A) Expression levels of HSPA4 in CRC tumor tissues and adjacent normal skin tissues were detected by IHC staining. (B) Kaplan-Meier survival analysis HSPA4 expression and overall survival of CRC. (C) HSPA4 expression in CRC cells was detected by qRT-PCR. (D) qRT-PCR was used to screen knockdown efficiency of HSPA4 in shHSPA4 (RNAi-1), shHSPA4 (RNAi-2), and shHSPA4 (RNAi-3). The data were presented as the mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.
Figure 2

The construction of HSPA4 knockdown cell model (A) Transfection efficiencies for HCT116 and RKO cells were evaluated by expression of green fluorescent protein 72 h post-infection. (B, C) The specificity and validity of the lentivirus-mediated shRNA knockdown of HSPA4 expression was verified by qRT-PCR (B) and Western Blot analysis (C). The data were presented as the mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.
Figure 3

Knockdown of HSPA4 inhibits cell proliferation, promotes apoptosis in CRC cells. (A) Cell proliferation of HCT116 and RKO cells with or without knockdown of HSPA4 was evaluated by Celigo cell counting assay. Flow cytometry analysis based on Annexin V-APC staining was utilized to detect cell apoptotic ratio (B) and cell cycle distribution (C) for HCT116 and RKO cells. The data were presented as the mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.
Figure 4

Knockdown of HSPA4 inhibits cell migration in CRC cells. Cell migration of HCT116 and RKO cells with or without knockdown of HSPA4 was evaluated by wound healing assay (A) and Transwell assay (B). The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.
Figure 5

Knockdown of HSPA4 inhibits tumor growth in mice xenograft models. (A) The volume of tumors in shCtrl group and shHSPA4 group was measured after post-injection. (B) The average weight of tumors in shCtrl group and shHSPA4 group. (C) Images of mice and tumors in shCtrl group and shHSPA4 group. (D) The total bioluminescent intensity of tumors in shCtrl group and shHSPA4 group. (E) The bioluminescence imaging of tumors in shCtrl group and shHSPA4 group. (F) The Ki67 staining of tumor tissues in shCtrl group and shHSPA4 group. The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.
Figure 6

Exploration of downstream molecular mechanism of HSPA4 in CRC cells (A) Human apoptosis antibody array analysis was performed in RKO cells with or without HSPA4 knockdown. (B) Densitometry analysis was performed and the gray values of differentially expressed proteins were shown. (C) The expression of downstream protein pathway was observed by Western Blot in RKO cells with or without HSPA4 knockdown. The data were presented as mean ± SD (n = 3), *P<0.05, **P<0.01, ***P<0.001.