Long non-coding RNA CASC11 interacts with hnRNP-K and activates the WNT/β-catenin pathway to promote growth and metastasis in colorectal cancer

Zheying Zhang a,b,1, Chang Zhou c,1, Yaya Chang a, Zuoyang Zhang a, Yuhan Hu a, Fan Zhang a, Yanxia Lu a, Lin Zheng a, Wenjuan Zhang a, Xiaomin Li a, Xuenong Li a,c,*

a Department of Pathology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China
b Department of Pathology, Xinxiang Medical University, Xinxiang, Henan, China
c Department of Anatomy and Histology, Guangdong Pharmaceutical University, Guangzhou 510006, China

ABSTRACT

The abnormal expression of many long non-coding RNAs (lncRNAs) has been reported in the progression of various tumors, and these lncRNAs can be useful as diagnostic indicators and anti-tumor targets. Therefore, it is important to identify lncRNAs that can be used for the clinical prevention and treatment of colorectal cancer (CRC). Here, we report that cancer susceptibility candidate 11 (CASC11) was upregulated in CRC tissues; increased CASC11 expression in CRC was associated with tumor size, serosal invasion, lymph metastasis, and the tumor–node–metastasis (TNM) stage. Functional experiments showed that CASC11 can promote CRC cell proliferation and metastasis in vitro and in vivo. Furthermore, CASC11 can target heterogeneous ribonucleoprotein K (hnRNP-K) to activate WNT/β-catenin signaling in CRC cells. In addition, we found that c-Myc directly bound to the promoter regions of CASC11 and increased promoter histone acetylation to enhance CASC11 expression. Together, our findings indicate that the novel lncRNA CASC11 may serve as a candidate diagnostic biomarker and a promising therapeutic target for CRC.

© 2016 Elsevier Ireland Ltd. All rights reserved.

Introduction

Colorectal cancer (CRC) remains a high-risk digestive tract tumor, and its incidence has increased in recent years; however, detailed molecular mechanisms for CRC remain unclear. Recently, multiple lines of evidence have revealed that long non-coding RNAs (lncRNAs) may be involved in tumorigenesis [1–3]. Therefore, it is imperative to identify CRC-related lncRNAs and investigate their functions in CRC.

Long non-coding RNAs (lncRNAs), non-protein-coding transcripts longer than 200 nucleotides, have emerged as essential regulators of cell growth and tumor metastasis that function by promoting or repressing transcription or by acting as modulators of mRNA translation [4]. For example, the lncRNA SchLAP1 promotes aggressive prostate cancer by antagonizing the SWI/SNF complex [5]. lncRNA ANRIL can recruit PRC2 to the P15 promoter and repress the expression of P15 [6]. PCAT1, a post-transcriptional repressor of the BRCA2 3’UTR, controls homologous recombination in prostate cancer [7]. LncRNA-p21 serves as a repressor in p53-dependent transcriptional responses through its physical association with hnRNPK [8].

Variants in the human 8q24 region have been associated with numerous cancers including CRC [9]. Many cancer-associated lncRNAs have been found in this 8q24 region. lncRNA CCAT2, mapping to 8q24, is overexpressed in CRC and can enhance tumor invasion and metastasis by regulating c-Myc transcription and activating the WNT signaling pathway [10]. lncRNA CCAT1 also maps to 8q24 and can enhance the progression of gastric carcinoma depending on the post-transcriptional activity of c-Myc [11]. The coamplification of PVT1 and c-Myc copy number promotes malignant pleural mesothelioma [12]. Previous studies have shown that some lncRNAs can affect the expression of the genes located in their chromosomal neighborhood [13,14]. Using NCBI data, we observed cancer susceptibility candidate 11 (CASC11) located in the chromosome 8q24 gene desert ~2.1 kb upstream of c-Myc. In addition, the sequence variant at 8q24 rs16902359 confers
susceptibility to lymphoma [15,16]. Additionally, we found that the CASC11 transcript encompasses the rs16902359 SNP. Therefore, we hypothesized that the lncRNA CASC11 may have a role in cancer.

Here, we found that CASC11 is overexpressed in CRC tissues and CRC-derived cell lines. Moreover, we found that CASC11 enhances cell growth and metastasis by targeting hnRNP-K, which can enhance β-catenin nuclear accumulation and activate WNT signaling. In addition, we showed that c-Myc can bind to the promotor regions of CASC11 and increase promotor histone acetylation to enhance CASC11 expression. Our results provide novel insights into the function of CASC11 in CRC pathogenesis as a regulator of WNT signaling that mediates this activity through hnRNP-K interaction.

Materials and methods

Cell culture

The cell lines FHC, LOVO, M5, HCT116, HT29, RKO, LS174T, SW480, SW620, and HEK293 were obtained from the American Type Culture Collection (ATCC) and authenticated according to the ATCC recommendations. Cells were cultured in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37 °C with 5% CO2.

Tissue samples and animals

Colorectal cancer specimens were collected at Nanfang Hospital, Southern Medical University (Guangzhou, China), and written informed consent was obtained from all patients. The tissue specimens were frozen in liquid nitrogen and stored at −80 °C. All tissues were confirmed as adenocarcinoma. Classifications were based on the system of the International Union Against Cancer. Male 4–6-week-old athymic BALB/c-nu-nu mice were purchased from and maintained at the Central Laboratory of Animal Science of Southern Medical University.

RNA extraction and qRT-PCR

Total RNA was extracted from tissues or cells using TRIzol reagent (Takara) according to the manufacturer’s instructions. RNA reverse transcription to cDNA was performed with a Reverse Transcription Kit (Takara). Quantitative real-time PCR (qRT-PCR) analyses used SYBR Green I (Takara) in triplicate. The ratios were normalized to the expression of GAPDH. The primer sequences used for qRT-PCR are listed in Supplementary Table S1.

Construction of plasmids and transfection

Lentiviral constructs repressing CASC11 were purchased from Genechem (Shanghai, China) and were used to establish cell lines constitutively repressing CASC11. The siRNA or shRNA nucleotide sequences for repressing CASC11, hnRNP-K, and c-Myc are listed in Supplementary Table S2. The siRNA used to inhibit hnRNP-K expression was purchased from GenePharma (Shanghai, China). shRNA hairpins against c-Myc were from Genechem (Shanghai, China). CASC11, hnRNP-K, and c-Myc expression plasmids were synthesized by Genechem (Shanghai, China). Cells were transfected with siRNA oligonucleotides and plasmids using Lipofectamine 2000 (Invitrogen).

Cell Proliferation, colony formation, and transwell assays

The proliferation, plate colony formation, invasion, and migration of transfected CRC cells were determined as previously described [17].

Flow cytometry

After 24 h, the transfected cells were harvested by trypsinization. Fluorescein isothiocyanate (FITC), Annexin V, and propidium iodide were used to stain 10^6 cells in G0–G1, S, and G2–M phases and counted and compared according to the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) protocol. A flow cytometric analysis was performed, and cells in G0–G1, S, and G2–M phases were counted and compared.

In vivo tumorigenic and metastasis assay

Male athymic BALB/c nu nude mice between 4 and 6 weeks of age were obtained from the Central Laboratory of Animal Science at Southern Medical University and maintained in laminar flow cabinets under specific pathogen-free conditions. All animal work was conducted according to national guidelines, and all animal experiments were approved by the ethical committee of the Southern Medical University. SW480-PLV-shCASC11 and SW480-PLV-shNC (1 × 10^6 cells per mouse) cells were injected subcutaneously into the left or right flank of nude mice (n = 5 per group). After 4 weeks, we analyzed primary tumor growth by the formula (length × width)^2/2 [18]. For the metastasis model, 2 × 10^5 cells were injected into the tail vein of nude mice (n = 5 per group); eight weeks later, the mice were sacrificed, and their liver and lung was removed and formalin fixed for histological analysis [19].

Immunohistochemistry (IHC)

The primary tumors were immunostained for hnRNP-K and Ki-67 as previously described [20].

RNA pull down assay

CASC11 and its antisense RNA were in vitro transcribed with T7/SP6 RNA polymerase, biotin-labeled using a Pierce RNA 3′ End Desthiobiotinylation Kit (Thermo), and treated with RNase-free DNase I (Thermo). The pull down assay was performed according to the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo). A quantity of 50 pmol biotinylated RNA was incubated with streptavidin agarose beads and mixed with protein; one hour later, washing and elution of RNA-Binding protein complexes was performed. The retrieved proteins were detected using a standard western blotting technique.

Western blot assay (WB)

Proteins were extracted by a lysis buffer and quantified using a bicinchoninic acid (BCA) protein quantification kit (KeyGen Biotech). Protein lysates were separated using 10% SDS-PAGE and transferred onto a PVDF membrane (Roche). Then, the membrane was incubated with specific primary antibodies followed by their respective appropriate second antibodies. The bands were visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific). Antibodies against c-Myc, β-catenin, AXIN, GSK3β, TC4, hnRNP-K, MMP7, and CyclinD1 were purchased from Abclone, Cambridge, MA, USA.

RNA immunoprecipitation (RIP)

RIP experiments were performed with the Magna RIP™ RNA-binding protein immunoprecipitation kit (Millipore) according to protocol. For the hnRNP-K, an antibody (Abclone) was used for RIP assays to co-precipitate RNA. RNA quantity was detected by qRT-PCR.

Subcellular fractionation

The separation of nuclear and cytosolic fractions was accomplished with the PARIS Kit (Life Technologies) according to the manufacturer’s instructions. An aliquot of 50 ng cDNA was added to KOD SYBR®qPCR Mix (TOYOBO) along with primers. Assays were run for 40 cycles using standard conditions on the Applied Biosystems 7500/7500 Fast (Applied Biosystems). Ct values were generated using SDS software [21].

TOP-Flash WNT reporter

The activity of the WNT pathway was examined using a TOP-Flash luciferase reporter. Cells were co-transfected with 250 ng TOP-FLASH or FOP-FLASH and 25 ng pRL-SV40 plasmid. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega); the ratios of TOP/FOP were calculated and used as indicators of WNT signaling activity.

Co-immunoprecipitation (CoIP)

Proteins were extracted from SW480 cells with lysis buffer. HNRP-K, β-catenin, GSK3β, or TC4 antibodies (Abclone) were added to cell lysates. Subsequently, approximately 30 μl of agarose–protein G beads was added. Beads were incubated for two hours, washed three times in PBS, and proteins were eluted in Laemmli buffer. Western blots were used to analyze the interacting proteins.

Luciferase activity assay

The pGL3-WT luciferase reporter plasmid was generated by ligating oligonucleotides containing a wild-type (WT) CASC11 promoter into the vector pGL3-basic (Promega). The pGL3-Mut plasmid with a mutant target site in the E-box was synthesized by Babiotech (Beijing, China). Cells were seeded in 24-well plates (1 × 10^4 cells per well) and cultured 24 hours before transfection. pGL3-basic, pGL3-WT, or pGL3-mutant plasmids were co-transfected with pRL-TK (Promega) and pCMV5-c-Myc using Lipofectamine 2000 (Invitrogen). pRL-TK (Promega) vectors were used as control. After 48 hours, luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed according to the EZ-ChIP kit (Millipore, Temecula, CA, USA). Anti-c-Myc (Abclone) or anti-H3K27AC (Abclone) antibodies were used
to precipitate the DNA-protein complexes. The immunoprecipitated DNA was examined by PCR. Primers specific for the CASC11 promoter containing the E-box were 5′-GCTGCCGGGTTTGGGAGAA-3′ (forward) and 5′-AGGACCTGGAAAGGAATT-3′ (reverse).

Statistical analysis

Statistical analyses were performed using SPSS20.0 software (IBM). Data are presented as the means ± standard deviation of at least 3 independent experiments. The differences between groups were tested using a two-tailed Student’s t-test. Relationships between CASC11 expression and clinicopathologic characteristics were determined by the $\chi^2$ test. Pearson’s correlation coefficient was used to measure the linear relationship between the expression levels of CASC11 and hnRNP-K or c-Myc in CRC tissues. Differences were considered significant if $p < 0.05$: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Results

CASC11 is up-regulated in colorectal cancer tissues and cells

To investigate the role of CASC11 in CRC tumorigenesis, the expression levels of CASC11 were detected in 36 paired CRC tissues and adjacent normal tissues by qRT-PCR. CASC11 expression was higher in 32 of 36 CRC specimens compared to the adjacent normal mucosa tissues ($p < 0.001$) (Fig. 1A and B). Furthermore, to investigate the clinicopathologic significance of CASC11, CASC11 expression was divided into a high-expression group ($n = 18$) and a low-expression group ($n = 18$) by the median CASC11 expression level. Correlation analysis showed that CASC11 expression was positively associated with tumor size, serosal invasion, lymph metastasis, and tumor–node–metastasis (TNM) stage in CRC (Table 1). We further evaluated the expression levels of CASC11 in the eight CRC cell lines LOVO, M5, LS174t, RKO, HT29, SW620, SW480, and HCT116. The expression levels of CASC11 were also up-regulated in these CRC cell lines compared with the normal colorectal epithelium cell line FHC. Error bars indicate the means ± SD of 3 independent experiments. *$p < 0.01$; ***$p < 0.001$.

CASC11 promotes proliferation and migration of colorectal cancer cell lines in vitro

To evaluate the roles of CASC11 in CRC, we knocked down CASC11 permanently with pLV-shCASC11 lentiviral particles in SW480 and SW620 cells. Controls were infected with empty lentivirus par-

Table 1  Clinicopathologic characteristics of CASC11 expression in CRC patients.

<table>
<thead>
<tr>
<th>Clinicopathologic Variables</th>
<th>N</th>
<th>High expression</th>
<th>Low expression</th>
<th>$X^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All case</td>
<td>36</td>
<td>18</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)$^a$</td>
<td>12</td>
<td>4</td>
<td>8</td>
<td>2.000</td>
<td>0.144</td>
</tr>
<tr>
<td>≤60</td>
<td>24</td>
<td>14</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>19</td>
<td>6</td>
<td>13</td>
<td>5.461</td>
<td>0.022</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Size (cm)$^b$</td>
<td>≤4.0</td>
<td>19</td>
<td>6</td>
<td>13</td>
<td>5.461</td>
</tr>
<tr>
<td>&gt;4.0</td>
<td>17</td>
<td>12</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>0.743</td>
</tr>
<tr>
<td>Moderate</td>
<td>23</td>
<td>12</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serosal Invasion</td>
<td>Yes</td>
<td>23</td>
<td>16</td>
<td>7</td>
<td>9.753</td>
</tr>
<tr>
<td>No</td>
<td>13</td>
<td>2</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph Metastasis</td>
<td>Yes</td>
<td>16</td>
<td>13</td>
<td>3</td>
<td>9.368</td>
</tr>
<tr>
<td>No</td>
<td>20</td>
<td>6</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM classification</td>
<td>I-II</td>
<td>17</td>
<td>5</td>
<td>12</td>
<td>5.461</td>
</tr>
<tr>
<td>III-IV</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Group of age was performed according to median.

*b* Tumor size was grouped according to median.
ticles. qRT-PCR assays showed that the expression levels of CASC11 were significantly reduced both in SW480 and SW620 cells (Supplementary Fig. S1A). CK8 proliferation assays revealed that CASC11 knockdown significantly decreased cell growth (Fig. 2A). Similarly, colony formation capacity was suppressed after the downregulation of CASC11 (Fig. 2B). Furthermore, the numbers of cells in G1 or G2/M phase was assessed by flow cytometry, and we found that pLV-shCASC11 cells had an proportion in the G1 phase indicative of a significant G1 phase cell cycle arrest compared with the pLV-shNC cells (Fig. 2C). The results of flow cytometry suggest that CASC11 regulates cell cycle-related proteins at the G1 phase. Decreased expression of CASC11 inhibits the cell cycle and attenuates the cell proliferation capacity in CRC. Cell motility was measured by transwell and wound healing assays, and the results revealed that the repression of CASC11 attenuated the migration of SW480 and SW620 cells (Fig. 2D and E). Moreover, we also generated SW620 and RKO cell lines overexpressing CASC11 (Supplementary Fig. S1B). Because we can obtain higher transfection efficiency in SW620 and RKO compared with other CRC cell lines, we used these lines to establish cell lines transiently overexpressing CASC11. As expected, SW620-pEGFP-C1-CASC11 and RKO-pEGFP-C1-CASC11 cells showed higher proliferative capacity (Supplementary Fig. S1C) and migratory ability compared with NC cells (Supplementary Fig. S1D).

Knockdown of CASC11 inhibits tumor proliferation and metastasis in vivo

To confirm the effect of CASC11 on tumor proliferation in vivo, SW480/shNC and SW480/shCASC11 cells were subcutaneously inoculated into the hind limb of nude mice. Tumor size was measured over time. Mice in the SW480/shCASC11 group developed smaller tumors than those in the SW480/shNC group (P < 0.01) (Fig. 3A and B). IHC assays confirmed that the Ki-67 proliferation index in the SW480/shCASC11–xenografted tumors was lower than that in SW480/shNC–xenografted tumors (Fig. 3C and D).

To determine the effect of CASC11 on CRC metastasis in vivo, SW480/shNC and SW480/shCASC11 cells were injected into the tail vein of mice. Eight weeks later, mice were killed, and lung and liver metastases were examined. None of the mice in the SW480/shCASC11 group had lung and hepatic metastatic nodules. However, 40% (2 of 5) of mice in the SW480/shNC group had lung metastatic nodules, and 80% (4 of 5) of these mice had hepatic metastatic foci (Fig. 3E). The number of hepatic metastatic nodules in mice of the SW480/shCASC11 group was obviously reduced compared with the SW480/shNC group (P < 0.05) (Fig. 3F). Consistent with the in vitro findings, these results suggest that CASC11 significantly promotes tumor growth and metastasis in vivo.

CASC11 associates with hnRNPK protein and increases its expression

Previous studies strongly suggested that IncRNA genes are involved in the regulation of signal pathways through their interaction with specific proteins [6,8,22,23]. RNA-pull-down assays were used to identify the CASC11 interacting proteins. We found several bands pulled down by vitro-transcribed biotinylated CASC11 sense transcript using silver staining. Heterogeneous ribonucleoprotein K (hnRNPK), one of the proteins identified by mass spectrometry, was confirmed by western blotting (Fig. 4A). hnRNPK is a protein with three K homology (KH) domains that mediate DNA and RNA binding, and it contains a nuclear shuttling domain (KNS) that mediates its transit between the nucleus and cytoplasm [24,25]. Next, we investigated CASC11 and hnRNPK binding in vivo by RNA-binding protein immunoprecipitation (RIP) experiments. We observed CASC11 enrichment using the hnRNPK antibody compared to a nonspecific antibody (IgG control) (Fig. 4B). Furthermore, we detected a downregulation of hnRNPK RNA and protein levels upon CASC11 repression in SW480 and SW620 cells. In contrast, we found higher hnRNPK expression at both the RNA and protein levels in cells overexpressing CASC11 (Fig. 4C). Previous study has shown that the biological roles of IncRNAs were related to their location in cells [26]. Thus, we examined the subcellular location of CASC11 in CRC cells and found a higher expression in cytosolic fractions compared with nuclear fractions (Fig. 4D). These results suggest that CASC11 may play an important role in regulating mRNA stability. To further determine whether CASC11 regulates the synthesis or degradation of hnRNPK mRNA, we enhanced or inhibited CASC11 expression in SW620 cells, treated the cells with actinomycin D (or dimethylsulfoxide control) over a 24 h period to block new RNA synthesis, and then measured the loss of hnRNPK-K, GAPDH, and 18s RNA over different periods [27]. We found that the inhibition of CASC11 decreased the stability of hnRNPK-K mRNA. Conversely, CASC11 overexpression increased the stability of hnRNPK-K (Fig. 4E). There was no change in the GAPDH mRNA level.

According to one report, the expression of hnRNPK in primary colorectal tumor samples was higher than in adjacent non-cancer tissue [28]. Consistent with this report, we observed that hnRNPK mRNA levels were significantly increased in colorectal cancer tissues compared to adjacent normal tissues (Supplementary Fig. S2A). In agreement with the qRT-PCR findings, IHC results showed an hnRNPK-K positive expression rate of 90% (36/40) in CRC tissues (Supplementary Fig. S2B). Correlation analysis indicated a positive correlation between CASC11 expression and hnRNPK-K expression (r = 0.570) (p < 0.001) (Fig. 4F). In addition, we found that knockdown of hnRNPK-K inhibited CASC11 mRNA levels, whereas hnRNPK-K overexpression boosted CASC11 transcription (Supplementary Fig. S2C). Therefore, we assume that CASC11 functions through its association with hnRNPK-K and that hnRNPK-K has a similar effect on CASC11. There may be a complex feedback loop between CASC11 and hnRNPK-K.

CASC11 directly targets hnRNPK to activate WNT signaling in CRC cells

According to a previous study, hnRNPK-K can interact with β-catenin to alter downstream gene expression and shuttling between the nucleus and cytoplasm [29]; β-catenin plays an important role in the activation of the WNT pathway [30]. Having previously shown that hnRNPK interacts with CASC11, we hypothesized that CASC11 could modulate Wnt/β-catenin signaling through its interaction with hnRNPK. Through gene set enrichment analysis (GSEA), we observed WNT signaling pathway correlation with CASC11 expression (Supplementary Fig. S3). To test the hypothesis that CASC11 plays an important role in activating WNT signaling, we used a TOP-Flash luciferase assay and observed that CASC11 downregulation inhibited WNT/β-catenin pathway activity. In contrast, CASC11 overexpression upregulated WNT/β-catenin signaling activity (Fig. 5A). We next detected the expression of downstream WNT pathway targets including β-catenin, c-Myc, CyclinD1, and MMP7. CASC11 inhibition decreased the expression of β-catenin, c-Myc, CyclinD1, and MMP7. However, CASC11 overexpression increased the expression of these proteins (Fig. 5B and C).

β-catenin is a transcriptional regulator of the canonical Wnt signaling pathway after it is imported into the nucleus [30]. To identify whether CASC11 increases the nuclear localization of β-catenin through hnRNPK-K, we altered the expression levels of CASC11 in SW480 and SW620 cells and examined the protein levels and nuclear translocation of β-catenin and hnRNPK-K. CASC11 overexpression increased the nuclear translocation of β-catenin and hnRNPK-K proteins. Conversely, CASC11 knockdown decreases the nuclear translocation of β-catenin and hnRNPK-K proteins. There were no obvious changes in the cytoplasmic levels of β-catenin and hnRNPK-K (Fig. 5D). We also assessed whether hnRNPK-K was necessary for the
Depletion of CASC11 in CRC cells inhibits cell growth and metastasis in vitro. (A) CASC11 knockdown inhibited cell proliferation on the basis of CCK8 assays. Error bars represent the means ± SD of 5 independent experiments. ***p < 0.001. (B) The colony formation assay used to evaluate the proliferation of SW480 and SW620 cells after inhibiting the expression of CASC11. The bar chart represents the colony number. Error bars represent the means ± SD of 3 independent experiments. ***p < 0.001. (C) Cell cycle was analyzed by flow cytometry after knocking down CASC11. The results showed that CASC11 depletion led to G1 arrest. (D) CASC11 inhibition decreased cell migration as determined by transwell assays. The bar chart represents the migration cell numbers. Error bars represent the means ± SD of 5 different fields. *p < 0.05; ***p < 0.001. (E) Wound healing assay used to determine the migration ability of cells with downregulated CASC11. The bar chart represents the percentage of distance at 24 h or 48 h divided by the distance at 0 h. Data are presented as the means ± SD of 3 independent experiments. **p < 0.01.
Fig. 3. Silencing CASC11 delays tumor growth and metastasis of CRC in vivo. (A) CASC11 knockdown inhibited subcutaneous tumor formation in a nude mouse model. SW480/shNC cells were subcutaneously inoculated into the right hind limb of nude mice. SW480/shCASC11 cells were inoculated into the left. (B) Comparison was made between the shNC group and the shCASC11 group at the weekly time points using t-tests. Data are the means ± SD for five samples. (C) The tumor sections were subjected to H&E and IHC staining using antibodies against Ki-67. (D) The Ki-67 index was calculated as the number of Ki-67 positive cells divided by the number of total cells × 100% (magnification, × 200). Error bars indicate the means ± SD of 5 different fields. Scale bars = 100 μm. (E) Representative image of hepatic and lung metastases in mice injected with SW480/shNC and SW480/shCASC11 cells (magnification, × 100). Scale bars = 200 μm. (F) The numbers of hepatic and lung metastases per mouse were calculated (n = 5). Comparison was made between the shNC group and the shCASC11 group using t-tests. Data are the means ± SD for five samples.
Fig. 4. CASC11 physically interacts with hnRNP-k and upregulates its expression. (A) RNA pulldown assays were used to identify associated proteins. Biotinylated CASC11 or antisense RNA was incubated with cell extracts, and associated proteins were resolved in a gel. After silver staining, specific bands were excised and identified by mass spectrometry (MS). hnRNP-K protein was assayed by WB. (B) RIP experiments were performed using an hnRNP-K antibody or non-specific IgG. The fold enrichment of CASC11 in hnRNP-K RIP is relative to input RNA. Data are presented as the means ± SD of 3 independent experiments. **p < 0.01. (C) hnRNP-K mRNA and protein levels after inhibiting CASC11 in SW480 and SW620 cells or after increasing CASC11 in SW620 and RKO cells. Error bars in the data from qRT-PCR represent the means ± SD of 3 independent experiments. ***p < 0.001. (D) SW480 and SW620 cells lysates were separated into cytoplasmic and nuclear fractions. We compared Ct values calculated from qRT-PCR with cDNA from the nuclear fraction to the Ct value from qRT-PCR using cDNA from the cytoplasmic fraction. GAPDH and U6 were used to assess the quality of cell fractionation. Error bars indicate the means ± SD of 3 independent experiments. (E) The stability of hnRNP-K and GAPDH mRNA was measured by qRT-PCR after blocking new RNA synthesis using actinomycin D (50μM) or dimethylsulfoxide (DMSO, negative control) and normalizing to 18S rRNA. Error bars represent the means ± SD of 3 independent experiments. *p < 0.05. (F) qRT-PCR analysis of CASC11 and hnRNP-K expression in 20 paired human colorectal cancer tissues and adjacent normal tissues; CASC11 and hnRNP-K expression was normalized to GAPDH, and the results are presented as the fold change in tumor tissues relative to the matched adjacent normal tissues. Spearman correlation analysis showed a positive relationship between CASC11 expression level and hnRNP-K mRNA in 20 CRC tissues.
Fig. 5. CASC11 enhances β-catenin nuclear accumulation to activate WNT signaling through hnRNP-K. (A) Cells were transfected with TOP-Flash or control FOP-Flash reporter to determine reporter activities 48 h later. The values of TOP-Flash and FOP-Flash were normalized to the value of pRL-SV40, and the bar chart presents the ratios of TOP/FOP. Values are the means ± SD for triplicate samples. *\( p < 0.05 \). (B) qRT-PCR was performed to detect CASC11, β-catenin, c-Myc, CyclinD1, and MMP7 expression after CASC11 upregulation or knockdown. Data are the means ± SD for triplicate samples. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \). (C) The expression of β-catenin, c-Myc, CyclinD1, and MMP7 in SW480 and SW620 cells were detected by WB after decreasing or increasing the expression of CASC11. β-tubulin served as the loading control. (D) The nuclear and cytoplasmic protein levels of hnRNP-K and β-catenin were determined by WB after inhibiting or promoting the expression of CASC11. β-tubulin served as cytosolic control, and H3 served as the nuclear control. (E) The nuclear protein levels of hnRNP-K and β-catenin were examined by WB after we inhibited the expression of CASC11 but increased the expression of hnRNP-K or increased the expression of CASC11 but inhibited hnRNP-K expression. β-tubulin served as the cytosolic control, and H3 served as the nuclear control. (F) Extracts of SW480 cells were subjected to COIP using hnRNP-K antibody or control IgG, and WBs were performed with β-catenin, Axin2, GSK3β, and TCF4 antibodies. Reciprocal COIPs were performed using β-catenin, Axin2, GSK3β, TCF4, and IgG antibodies, followed by WB with hnRNP-K antibody.
CASC11-induced nuclear localization of β-catenin. The reintroduction of hnRNP-K in CASC11-depleted cells increased the nuclear translocation of β-catenin and hnRNP-K. Moreover, the re-depletion of hnRNP-K abolished the promoting effect of CASC11 (Fig. 5E). These data suggested that CASC11 increases β-catenin nuclear accumulation in an hnRNP-K-dependent manner.

To further ascertain the role of hnRNP-K in β-catenin nuclear accumulation, we tested whether hnRNP-K could interact with β-catenin, AXIN, GS3Kβ, and TCF4 to form a complex by co-immunoprecipitation (COIP). Because the components AXIN and GS3Kβ induce the degradation of β-catenin, the interaction between hnRNP-K and β-catenin, AXIN2, and GS3Kβ suggested that hnRNP-K might protect β-catenin from degradation [31]. A previous study has shown that hnRNP-K can shuttle between the nucleus and cytoplasm [24]. Moreover, we found that there was an interaction between hnRNP-K and TCF4 (Fig. 5F). These results suggested that β-catenin nuclear accumulation activates WNT signaling through complexation with hnRNP-K. Whether CASC11 also forms a complex with hnRNP-K and β-catenin in this process and our hypothesis concerning the role of hnRNP-K in β-catenin nuclear accumulation need more experiments to confirm.

c-Myc directly binds to the promoter regions of CASC11 and increases promoter histone acetylation to enhance CASC11 expression

To examine how the transcription of CASC11 is regulated, we analyzed the potential transcription factor binding sites in the promoter regions of CASC11 (http://www.gene-regulation.com/index2) and found one E-box element that is a candidate c-Myc binding site. c-Myc effectively stimulated the luciferase activity of the CASC11 promoter in HEK293 and SW480 cells (Fig. 6A). Additionally, ChIP assays showed that c-Myc directly binds to this region (~1274 to 1279 bp) in the CASC11 promoter (Fig. 6B). Moreover, cell lines with ectopic c-Myc expression showed higher CASC11 expression levels. In contrast, c-Myc knockdown decreased the expression of CASC11 in SW480 and SW620 cells (Fig. 6C). Finally, we also found that CASC11 expression was positively correlated with c-Myc in CRC tissues (Fig. 6D). These results indicated that c-Myc could directly bind to the E-box element and regulate the expression of CASC11.

We performed a computational screen of the promoter regions of CASC11 (https://genome.ucsc.edu/cgi-bin/hgGateway) and found an obvious Histone H3 Lysine 27 acetylation (H3K27AC) region in the promoter of CASC11 (Fig. 6E). CHIP assays confirmed that acetylation occurred in the CASC11 promoter (Fig. 6F). Previously, histone acetyltransferases (HATs) were found to be recruited by c-Myc [32]. To verify that c-Myc can enhance the acetylation of CASC11 promoter, we knocked down c-Myc in SW620 cells and found that inhibition of c-Myc decreased the level of H3K27AC in the E-box region of the CASC11 promoter (Fig. 6G). To further delineate the role of histone acetylation in the mediation by c-Myc of CASC11 transcriptional induction, we knocked down c-Myc in SW620 cells and treated them with the histone deacetylase (HDAC) inhibitor TSA. TSA increased the expression of CASC11 in sh-c-Myc cells (Fig. 6H). These results suggest that c-Myc increases promoter histone acetylation to enhance CASC11 expression.

Discussion

It is becoming clear that mammalian genomes encode numerous IncRNAs [13]. Many IncRNAs are reported to play an important role in cell biology, including the regulation of tumor progression [33]. It is notable that the 8q24 gene desert is a desert for protein-coding genes but not for IncRNAs [34]. Several IncRNAs are located in the 8q24 gene desert, and all play an important role in tumor progression [79–123,35]. CCAT1 is reported to be located in the super-enhancer region and to regulate c-Myc expression [36]. In addition, CCAT1 can also be regulated by c-Myc [37]. CCAT2 can enhance WNT signaling activity and regulate c-Myc expression [10]. PCAT1, an IncRNA mapping to 8q24, regulates BRCA2 post-transcriptionally in sporadic cancer [7]. PCAT1 has also been reported to promote prostate cancer cell proliferation by regulating c-Myc [38]. CASC11 is a novel IncRNA located in the 8q24 gene desert approximately 2.1 kb upstream of c-Myc. To date, there are no reports of CASC11 having specific roles in cancer. We find that CASC11 encompasses the rs16902359 SNP, which is associated with lymphoma [15,16]. Although the chromosomal region of CASC11 may provide some clues about its functional role, its general function and underlying mechanisms in CRC have remained elusive and require further investigation. Primarily, it is necessary to explore the expression patterns of CASC11 in normal human and CRC tissues. Here, we report that CASC11 is pathologically upregulated in CRC cells and tissues. Moreover, CASC11 upregulation in CRC patients is associated with tumor size, serosal invasion, lymph metastasis, and TNM classification. Our results indicate that CASC11 overexpression may be a common incident in CRC, and CASC11 seems to function as an oncogene during CRC genesis and progression.

The association of CASC11 with CRC development has not previously been reported. In this study, we performed systematic shRNA knockdown and overexpression experiments to assess the role of CASC11. We observed that the inhibition of CASC11 suppressed the proliferative and metastatic capacity of CRC cells. Conversely, CASC11 upregulation promoted CRC cell growth and invasion. In addition, xenograft tumor assays provided support for the involvement of CASC11 in enhancing CRC cell growth and metastasis in vivo. Thus, our data further confirmed the hypothesis that CASC11 exerts oncogenic activity to promote cell proliferation and metastasis in CRC.

The mechanism of CASC11 in promoting CRC progression remains uncertain. Recent studies have shown that IncRNAs interact with proteins to perform their function [6,8,22,23]. Thus, we presumed that the role of CASC11 in CRC might also be mediated by such a mechanism. To verify this hypothesis, we performed RNA pull-down assays and MS analysis. The results indicated interaction between CASC11 and the hnRNP protein hnRNP-K. The association is further supported by RIP assay. hnRNP-K is a protein with three K homology (KH) domains that can bind to DNA or RNA, and it contains a nuclear shuttling domain (KNS) that mediates its transit to the nucleus, where it can play an important role in transcription, mRNA transport, and stability [24,25]. We further showed through overexpression and knockdown experiments that CASC11 could regulate the expression level of hnRNP-K. According to previous study, the biological roles of IncRNAs are related to their subcellular localization [26]. Nuclear IncRNAs primarily regulate the transcription of target genes, whereas cytoplasmic IncRNAs may function as cytoplasmic scaffolds of RNA-protein complexes and may associate with target mRNAs to regulate mRNA stability and translation [39–43]. Through subcellular fractionation assays, we found that CASC11 was mainly localized in the cytosol of CRC cells. Then, we showed that the stability of hnRNP-K could be regulated by CASC11, which was supported by data showing that cytoplasmic IncRNAs could enhance the stability of target genes to increase their expression levels [27,44]. Moreover, when we altered the expression level of hnRNP-K, CASC11 expression changed correspondingly. The expression of hnRNP-K in CRC tissues also showed a similar trend with CASC11 RNAs. This observation was consistent with previous studies reporting increased hnRNP-K levels in cancerous tissues, and it provided further support for the tumor-promoting role of hnRNP-K [28]. The correlation analysis indicated a statistical correlation between CASC11 and hnRNP-K. We hypothesize that there may be a positive feedback loop between CASC11 and hnRNP-K. Therefore, we speculate that the complex of CASC11 and hnRNP-K could function to activate target downstream genes.
Fig. 6. c-Myc directly binds to the promoter regions of CASC11 and increases promoter histone acetylation to enhance CASC11 expression. (A) A schematic of the CASC11 promoter luciferase construct is depicted with the locations of the E-box element and the sequences of mutation. The relative luciferase activities of the WT or Mut E-box regions in HEK293T and SW480 cells were examined after transfecting with c-Myc 48 hour later. The values of PGL3-WT and PGL3-Mut are relative to PGL3-basic. Data are the means ± SD for triplicate samples. ***p < 0.001. (B) c-Myc binding at the promoter region of CASC11 containing the E-box element was assessed by CHIP assay. (C) CASC11 RNA levels after transfecting the c-Myc expression vector or sh-c-Myc into the SW480 and SW620 cells. CASC11 expression was normalized to GAPDH. Error bars indicate the means ± SD of 3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. (D) qRT-PCR analysis of c-Myc and CASC11 expression in 20 paired human colorectal cancer tissues and adjacent normal tissues; c-Myc and CASC11 expression was normalized to GAPDH, and the results are presented as the fold change in tumor tissues relative to the matched adjacent normal tissues. Spearman correlation analysis showed a positive relationship between CASC11 expression level and c-Myc mRNA in 20 CRC tissues. (E) The promoter region of CASC11 exhibited strong characteristics of histone H3 acetylation. Histone modification data were retrieved from the ENCODE collection. (F) H3k27AC at the promoter region of CASC11 was assessed by CHIP using primers to the E-box element. (G) SW620 cells were transfected with sh-c-Myc plasmid or control plasmid, and 48 h later, the acetylation level was examined by CHIP. (H) CASC11 expression levels were determined by qRT-PCR in SW480 and SW620 cells transfected with c-Myc or exposed to the HDAC inhibitor TSA. Data are the means ± SD for triplicate samples. **p < 0.01; ***p < 0.001.
The abnormal activation of the WNT/β-catenin signaling pathway is widespread in human cancers including CRC. Most CRCs are associated with aberrant WNT/β-catenin signaling activity [45]. As is known, hnRNP-K can interact with β-catenin to alter downstream gene expression and shuttle it between the nucleus and cytoplasm [29]. Consistently, we observed by COIP that hnRNP-K associates with β-catenin. β-catenin plays an important role in the activation of the WNT/β-catenin pathway [30]. Through gene set enrichment analysis (GSEA), we found that the WNT/β-catenin signaling pathway was correlated with CASC11 expression. We further confirmed that CASC11 upregulated WNT/β-catenin signaling activity using a TOP-Flash luciferase assay. These findings suggest that CASC11 may target hnRNP-K to activate WNT signaling.

Although our findings link CASC11 to the WNT/β-catenin pathway, the mechanism of CASC11 in WNT signaling activation is not currently understood in depth. β-catenin is a key modulator of WNT signaling transduction mediated through its nuclear localization [30] or β-catenin biosynthesis [46]. Our results showed that CASC11 upregulation increased the expression levels of β-catenin and hnRNP-K. Moreover, CASC11 upregulation was accompanied with β-catenin and hnRNP-K nuclear accumulation, and this process could be suppressed by re-inhibiting the expression of hnRNP-K. According to our previous MS data, CASC11 can interact directly with hnRNP-K but not with β-catenin. Therefore, we concluded that CASC11 increased β-catenin nuclear accumulation to accelerate WNT signaling by forming a complex with hnRNP-K. In addition, we found that hnRNP-K could interact with AXIN and GSK3. Because AXIN2 and GSK3β increase the degradation of β-catenin [31], the interaction between hnRNP-K and β-catenin, AXIN2, and GSK3β suggest that hnRNP-K may protect β-catenin from degradation. A previous study has shown that hnRNP-K shuttles between the nucleus and cytoplasm [24], and we found an interaction between hnRNP-K and TCF4. TCF4 is located in the nucleus, and the interaction between hnRNP-K and TCF4 indicated that hnRNP-K may play a role as a nuclear transport protein in β-catenin nuclear accumulation. These results suggest that hnRNP-K has an important role in β-catenin nuclear transport. Whether CASC11 maintains a complex with hnRNP-K and β-catenin during the whole process and the specific role of hnRNP-K in transporting β-catenin to the nucleus require further studies to elucidate the complete regulatory mechanism.

Recent studies showed that lncRNAs located in the 8q24 gene desert can be activated by c-Myc to promote the progression of tumors [9,11,38]. In the present study, we found using NCBI data that the nearest gene to c-Myc, ~2.1-kb upstream, was CASC11. Our results demonstrated that c-Myc can directly bind to the E-box element in the CASC11 promoter region and regulate the expression of CASC11. Through computational screening of the promoter regions, we found an obvious H3k27AC region in the promoter of CASC11. A recent study showed that c-Myc recruited HATs to the H19 promoter, leading to the activation of the promoter to upregulate H19 expression [32]. In our results, c-Myc can increase the histone acetylation level in the promoter region of CASC11, and histone acetylation plays an important role in regulating CASC11 expression. Thus, we concluded that c-Myc upregulates the CASC11 expression level by enhancing promoter the histone acetylation of CASC11.

From the above results, we can explain why hnRNP-K counteracts CASC11. CASC11 can increase the expression of hnRNP-K; then, hnRNP-K promotes β-catenin nuclear accumulation to activate WNT signaling. After WNT pathway activation, the expression of the downstream gene c-Myc was also activated. c-Myc, as a transcription factor, can promote histone acetylation to increase CASC11 expression. Finally, we demonstrate that there exists a positive feedback loop between CASC11 and hnRNP-K.

Interestingly, CCAT2, an lncRNA mapping to 8q24, can regulate the transcription of c-Myc and activate WNT signaling to promote cancer growth and metastasis in CRC [10]. The mechanism of CCAT2 was similar to CASC11 in CRC. Furthermore, the lncRNA CCAT1, which is located in 8q24, was also regulated by c-Myc [47]. We found that these lncRNAs have the same role and mechanism in cancer. Because
they are located in the same area, we think that their regulation may be similar to the INK4b-ARF-INK4a tumor suppressor locus: all for one or one for all [48].

In summary, we show that a novel lncRNA CASC1 is an important diagnostic factor for CRC and that it promotes CRC cell proliferation and metastasis in vitro and in vivo. CASC1 participates in the development and progression of CRC by associating with hnRNP-K and activating WNT/β-catenin signaling. Our findings indicate that CASC11 may be a promising therapeutic target in CRC.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (No. 81272758, No. 81302158, No. 81502479) and the Natural Science Foundation of Guangdong Province, China (No. 2014A030310099).

Conflicts of interest

We declare no competing financial interests in our study.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.03.022.

References
