LncRNA HOTTIP mediated DKK1 downregulation confers metastasis and invasion in colorectal cancer cells

Authors: Yiqi Rui, Mingchao Hu, Peng Wang, Chuanqiang Zhang, Hua Xu, Yuanzhong Li, Yu Zhang, Jianchun Gu and Qiang Wang

DOI: 10.14670/HH-18-043
Article type: ORIGINAL ARTICLE
Accepted: 2018-09-19
Epub ahead of print: 2018-09-19

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed. Pre-print author’s version
LncRNA HOTTIP mediated DKK1 downregulation confers metastasis and invasion in colorectal cancer cells

Yiqi Rui¹, Mingchao Hu¹, Peng Wang¹, Chuanqiang Zhang¹, Hua Xu¹, Yuanzhong Li¹, Yu Zhang¹, Jianchun Gu¹, Qiang Wang¹*

¹Department of General Surgery, Affiliated Jiangsu Shengze Hospital of Nanjing Medical University, Jiangsu 215000, China.

*Corresponding author: Qiang Wang, E-mail: jsszyy_wangqiang@163.com, Tel: +86-18906256023, Fax: +0512-63097011

Abstract

Recent studies highlight long non-coding RNAs (lncRNAs) as key regulators of cancer biology that contribute to carcinogenesis. The lncRNA HOXA transcript at the distal tip (HOTTIP) is involved in the development of several cancers. Previous studies demonstrated that HOTTIP could promote colorectal cancer (CRC) cell proliferation via silencing of p21 expression. However, the potential role of HOTTIP in CRC metastasis has not yet been discussed. Here, we found that HOTTIP level was significantly higher in CRC than in corresponding adjacent normal tissues, and patients with a larger tumor size, advanced pathological stage, or distant metastasis had higher HOTTIP expression. Moreover, silencing HOTTIP expression by siRNA or shRNA could inhibit CRC cell migration and invasion in vitro and in vivo, whereas HOTTIP overexpression promoted cell metastasis, as documented in the SW480 cell lines. Mechanistic analyses indicated that HOTTIP regulates CRC cell metastasis partly through the downregulation of tumor suppressor DKK1 expression. Collectively, our results suggest that tumor expression of lncRNA HOTTIP plays an important role in CRC metastasis. HOTTIP may serve as a candidate biomarker in this disease.

Keywords: colorectal cancer, metastasis, HOTTIP, DKK1, lncRNA
Introduction

Colorectal cancer (CRC) is the third most common malignancy and the fourth most frequent cause of cancer-related deaths worldwide, with particularly high incidence in Western countries (Favoriti et al., 2016; Torre et al., 2015). Although survival rates of CRCs are improving, metastasis is the major cause of death in patients with CRC, and more than one-third of patients with CRC ultimately develop metastatic disease spread (Kopetz et al., 2009). Similar to most other malignancies, lack of biological and molecular markers for cancer cell metastasis is still one of the most important obstacles challenging metastatic CRC therapy (Fung et al., 2014; Shah et al., 2014). Therefore, new findings on diagnostic and prognostic biomarkers associated with CRC progression and metastasis would be of great clinical relevance.

With the rapid development of molecular biology, long non-coding RNA (lncRNA) has caused great interest because of its essential role in a diverse range of tumor cellular processes such as proliferation, apoptosis, and cell metastasis (Schmitt and Chang, 2016; Schmitz et al., 2016). Briefly, lncRNAs are a class of non-protein-coding transcripts that are longer than 200 nucleotides and are transcribed by RNA polymerase II (RNA pol II) (Shi et al., 2013). Also, accumulating evidence has suggested that lncRNAs may promote the progression and metastasis of CRC (Han et al., 2015). For example, PVT-1, which encodes a long non-coding RNA, generates antiapoptotic activity in CRC, and dysregulation of PVT-1 was a prognostic indicator for CRC patients (Takahashi et al., 2014). CRC-associated lncRNA (CCAL) is an oncogenic lncRNA that promotes the tumorigenesis, MDR, and progression of CRC by activating a Wnt/β-catenin signaling pathway via suppression of activator protein 2α. The long non-coding RNA Taurine upregulated gene 1 (TUG1) participates in colorectal tumorigenesis and promotes metastasis by affecting epithelial-mesenchymal transition (Sun et al., 2016). Therefore, identification of CRC-associated lncRNAs and their associated molecular mechanisms is necessary for understanding progression and establishing better treatment of CRC.
Dickkopf WNT signaling pathway inhibitor 1 (DKK1) is an inhibitor of the Wnt/β-catenin signaling pathway (MacDonald et al., 2009). The tumor-suppressing function of DKK1 has been demonstrated in a colon cancer model, and low expression of DKK1 has been reported in CRCs (Maehata et al., 2008). In addition, several studies have demonstrated that DKK1 could inhibit the migration and invasion of breast cancer (Cowling and Cole, 2007; Sato et al., 2010; Xu et al., 2012). Recently, it has been gradually verified that dysregulation of DKK1 is potentially involved in the biological function of several cancer-related non-coding RNAs (Cowling and Cole, 2007; Sato et al., 2010; Xu et al., 2012).

The lncRNA HOXA transcript at the distal tip (HOTTIP), a long non-coding RNA that consists of more than 4,000 nt, is located on chromosome 7p15.2 (Wang et al., 2011). LncRNA HOTTIP has been reported to function as an oncogene in many types of human cancers (Cheng et al., 2015; Quagliata et al., 2014; Tsang et al., 2015; Zhang et al., 2015). In a previous study, Lian et al found that abnormal expression of HOTTIP was an unfavorable indicator for CRC patients and that silencing HOTTIP increased CRC cell growth arrest and induced apoptosis via upregulation of p21 expression (Lian et al., 2016). However, the significance of HOTTIP in CRC metastasis is still unknown. The goal of our study was to investigate the expression and metastasis function role of HOTTIP in CRC. Furthermore, we also tried to understand the relationship between HOTTIP and DKK1 in CRC, and seek the biological role and potential molecular mechanisms of HOTTIP on metastasis and the invasion of CRC, and to find a potential therapeutic target for CRC patients with metastases.
Materials and methods

Clinical tissue samples

A total of 53 paired CRC and adjacent normal tissues were obtained from patients with CRC who had undergone surgery at the affiliated jiangsu shengze hospital, Nanjing Medical University, from 2011 to 2017. All cases were confirmed as CRC based on histopathological evaluation. No prior treatment of these patients had been conducted before surgery. Clinicopathological characteristics were recorded in detail. All collected tissue samples were rapidly snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Informed consent was obtained from all patients. Our study was approved by the Research Ethics Committee of Nanjing Medical University, China.

Total RNA isolation and qPCR assay

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA quantity and quality were determined by NanoDrop2000c (Thermo Scientific, Waltham, MA, USA). For qRT-PCR, 1 µg of RNA was reverse-transcribed to cDNA using a Reverse Transcription Kit (Takara, Dalian, China). The qRT-PCR assays were conducted on an ABI 7500. Data were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used for target amplification are listed in Additional Files. All qRT-PCR data were calculated and expressed relative to the threshold cycle (shown as ΔCT) values and then converted to fold changes.
Cell lines and culture conditions

Five CRC cell lines (HT29, SW480, SW620, Caco2, and HCT116) were obtained from American Type Culture Collection (Manassas, VA, USA). All of the cell lines were grown and maintained in a RPMI 1640 or DMEM (GIBCO-BRL) medium (Invitrogen) and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Shanghai, China) at 37°C with 5% CO₂.

Cell transfection

Small interfering RNA (siRNA) and si-NC were synthesized (Carlsbad, California, USA) and transfected into cells using Lipofectamine 2000 (Invitrogen, USA). To overexpress HOTTIP, the full-length coding sequence for HOTTIP was amplified and subcloned into the pcDNA 3.1(+) vector (Invitrogen) according to the manufacturer’s instructions. SW480 cells were transfected with an empty vector or the HOTTIP-expressing plasmid according to the manufacturer’s protocol. After 48 hours of transfection, the cells were harvested for further study. The sequences of the siRNAs are described in Additional Files.

Cell viability and colony formation assay

Cell viability was monitored using a Cell Proliferation Reagent Kit I (MTT; Roche Applied Science). The SW480 cells were transfected with si-HOTTIP or si-NC (3,000 cells/well) and were cultured in 96-well plates with six replicate wells. Cell viability was assessed according to the manufacturer’s recommendations. For the colony formation assay, a total of 500 cells were placed in a six-well plate and maintained in a medium containing 10% FBS, which was replaced every five days. After two weeks, cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich). Visible colonies were manually counted. Triplicate wells were measured in each
Wound healing assay

For the wound healing assay, $3 \times 10^5$ cells were seeded in six-well plates, cultured overnight, and transfected with si-HOTTIP, si-NC, an empty vector, or pcDNA-HOTTIP. Once the cultures reached 90% confluence, the cell layer was scratched with a sterile plastic tip and washed with culture medium. After 24 hours, images of the plates were acquired using a microscope. The distance between the two edges of the scratch was measured using the Digimizer software system. The assay was independently repeated three times.

Transwell assay

For the migration assays, at 48 hours post-transfection, $1 \times 10^5$ cells in serum-free media were placed into the upper chamber of an insert (8-µm pore size; Millipore). For the invasion assays, $1 \times 10^5$ cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 hours, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, and the migrated and invasion cell numbers and percentages were then counted using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were independently repeated three times.

Western blot and antibodies

After 48 hours of transfection, SW480 cells were lysed with RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with a protease inhibitor cocktail.
Protein extracts (40 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to 0.22-µm nitrocellulose membranes (Sigma) and incubated with anti-DKK1 antibody. GAPDH antibody was used as a control. GAPDH antibody was purchased from Sigma-Aldrich (USA, cat. no. G8795) and anti-DKK1 antibody was purchased from Abcam (cat. no. ab109416). ECL chromogenic substrate was used and signals were quantified by densitometry (Quantity One software, Bio-Rad).

**In vivo tumor metastasis assays**

Four-week-old male athymic mice were purchased from the Animal Center of the Nanjing University (Jiangsu, China) and maintained in pathogen-free conditions. SW480 cells transfected with sh-HOTTIP or an empty vector were harvested from six-well plates, washed with phosphate-buffered saline (PBS), and resuspended at a density of 2 × 10⁷ cells/ml. The cell suspension (0.1 ml) was injected into the tail veins of five mice, which were euthanized six weeks after the injection. The lungs were removed and photographed, and visible tumors on the lung surface were counted. This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The sequence for HOTTIP shRNA is also listed in Additional Files.

**Statistical analysis**

All statistical analyses were performed using SPSS software, version 22.0 (SPSS, Chicago, IL, USA). The Student’s t-test or the chi-squared test was used to evaluate significant differences between groups of data. All data are represented as the means ± SD. Differences were considered significant if \( P < 0.05 \). “*” indicates \( P < 0.05 \); “**” indicates \( P < 0.01 \).
Results

Upregulation of HOTTIP is correlated with CRC progression and metastasis

HOTTIP expression levels were detected in 53 paired CRC samples and adjacent normal tissues by qRT-PCR and normalized to GAPDH. Similar to Lian et al previous study (Lian et al., 2016), the results showed that HOTTIP expression levels were remarkably higher in the tumor tissues than in the adjacent normal tissues ($P<0.01$; Fig. 1a). Next, in order to investigate the relationship between HOTTIP expression and clinical pathological features, we divided the samples into high (above the median, $n=27$) and low (below the median, $n=26$) HOTTIP expression groups according to the median value of HOTTIP levels. A chi-square test was then performed to evaluate the clinicopathological features between the two groups. As shown in Fig. 1b, 1c, and 1d, increased HOTTIP expression levels in CRC were significantly correlated with larger tumor sizes ($P=0.028$), advanced TNM stages ($P=0.025$), and distant metastasis ($P=0.035$). However, several other clinical parameters were found to not be significantly associated with HOTTIP expression in this study (Table 1).

Modulation of HOTTIP expression in CRC cells

We next investigated the level of HOTTIP in CRC cell lines, including SW620, HCT116, HT29, Caco2, and SW480. We found that there were higher expression levels of HOTTIP in SW480 and Caco2 cells compared with other CRC cells (Fig. 2a). So we firstly inhibited the endogenous expression of HOTTIP both in SW480 and Caco2 cells by siRNA to further investigate the functional effects of HOTTIP in CRC cells. To exclude off-target effects, we designed three different siRNAs, namely, si-HOTTIP, si-HOTTIP 2, and si-HOTTIP 3. Subsequently, si-NC, si-HOTTIP, si-HOTTIP 2, and si-HOTTIP 3 were transfected into the SW480 and Caco2 cell lines. qPCR assays revealed that HOTTIP expression was reduced in all si-HOTTIP cells, si-HOTTIP 2 cells, and si-HOTTIP 3 cells compared with control cells (si-NC). To
ensure efficient knockdown, we used only si-HOTTIP in further studies ($P<0.01$; Fig. 2b). Moreover, HOTTIP was overexpressed in SW480 cells using transfection of pcDNA-HOTTIP compared with SW480 cells transfection using an empty vector ($P<0.01$; Fig. 2c).

**Knockdown HOTTIP suppresses CRC cell migration and invasion**

To investigate the roles of HOTTIP in CRC cells, we performed an MTT assay. The results showed that knockdown of HOTTIP expression significantly inhibited cell viability in SW480 cell lines compared with control cells ($P<0.01$; Fig. 3a). Similarly, the result of colony formation assays revealed that clonogenic survival was strikingly decreased following inhibition of HOTTIP in SW480 cell lines ($P<0.01$; Fig. 3b). Next, to evaluate the effect of HOTTIP on the migration and invasion of SW480 cells, wound healing and Transwell assays were performed to determine the migratory and invasive abilities of cells after transfection with siRNA against HOTTIP. The wound healing assay revealed that the migratory ability of SW480 cells in the si-HOTTIP group was lower at 24 hours post-wound compared with si-NC ($P<0.05$; Fig. 3c). It was also observed via Transwell migration assay that the migration capacity of SW480 and Caco2 cells was significantly suppressed when HOTTIP was silenced by siRNA ($P<0.01$; Fig. 3d, f). The cell invasion assay showed that the number of invading cells was significantly lower in the si-HOTTIP group than in the control group in SW480 and Caco2 cells ($P<0.01$, Fig. 3e, g). Taken together, these findings suggest that HOTTIP may be closely associated with invasion and migration capacity of CRC cells.

**Overexpression of HOTTIP promotes CRC cell migration and invasion**

Considering that silencing HOTTIP could significantly impair the ability of CRC cells’ migration and invasion, we next transfected the SW480 cell line with
pcDNA-HOTTIP and detected the effect of overexpressed HOTTIP on CRC cell metastasis capacity. As expected, the results showed that HOTTIP overexpression promoted SW480 cells’ viability both in MTT and colony formation ($P<0.05$; Fig. 4a, 4b) and caused a significant facilitation of cell migration in a wound-healing assay ($P<0.05$; Fig. 4c). Moreover, we also carried out the Transwell assay to assess the contribution of HOTTIP to cell migration and invasion. As shown in Figs. 4d and e, overexpressed HOTTIP increased migration of SW480 cells and enhanced cell invasion ability compared with control groups ($P<0.05$).

**Silencing HOTTIP inhibits CRC cell metastasis in vivo**

To further explore the role of HOTTIP on the metastasis of CRC cells in vivo, we evaluated the effect on metastasis six weeks after the mice had been injected with SW480 cells transfected with sh-HOTTIP or an empty vector through the tail vein. We examined the number and size of tumor metastatic nodules in the lung. As shown in Figs. 5a and 5c, the number of pulmonary metastatic nodules was clearly decreased in the knockdown of HOTTIP group compared with that of the control group ($P<0.05$). This difference was further confirmed following examination of the entire lungs, and through HE staining of lung sections. These results indicate that silencing HOTTIP expression could inhibit tumor metastasis in vivo.

**Inhibition of Dickkopf-1 (DKK1) is partly involved in the oncogenic function of HOTTIP**

To explore the molecular mechanisms by which HOTTIP contributes to the phenotypes of CRC cells, we investigated potential targets involved in tumor metastasis. A Heat map showed expression levels of 10 different expressed transcripts in SW480 cells with knockdown of HOTTIP expression for 48 hours (Figs. 6a). We examined mRNA levels of metastasis-associated and EMT-associated genes (such as
E-cadherin, N-cadherin, vimentin, DKK1, EMP1, and FN1) in SW480 cells with knockdown of HOTTIP expression. Interestingly, DKK1 was found to be markedly altered among them. When HOTTIP was blocked or overexpressed, DKK1 mRNA was elevated 2.5-fold or diminished by 45%, respectively, compared to the control groups ($P<0.05$; Figs. 6b, 6c). In addition, expressions of metastasis-associated and EMT-associated genes including E-cadherin, N-cadherin, vimentin, EMP1, and FN1 were not affected by suppressing or overexpressing HOTTIP in SW480 cells (Figs. 6b, 6c). It has been confirmed that DKK1 is misregulated in a variety of human cancers and closely associated with tumor metastasis (Menezes et al., 2012). And a recent study indicated that DKK1 could inhibit epithelial-mesenchymal transition of colon cancer cells and contribute to colon cancer suppression (Qi et al., 2012). Therefore, we determined the expression of the DKK1 protein by performing western blot analysis. As expected, The DKK1 protein level was also elevated 2.0-fold in SW480 cells transfected with si-HOTTIP and reduced by approximately 60% after transfection of the cells with pcDNA-HOTTIP compared to the respective controls (Fig. 6d). These data indicate that DKK1 is negatively regulated by HOTTIP at the mRNA and protein levels. To further investigate whether DKK1 was involved in the HOTTIP-induced CRC cell migration and invasion, we carried out rescue experiments. SW480 cells were co-transfected with HOTTIP and DKK1 siRNAs. Colony formation assay and Transwell assay results indicated that co-transfection could partially rescue si-HOTTIP-inhibited metastasis in SW480 cells (Figs. 7b, 7c). Taken together, these findings suggest that HOTTIP promotes CRC cell migration and invasion partly through downregulation of DKK1 expression.

**Discussion**

Although stage I CRC patients have a five-year relative survival rate greater than 90%, most CRC patients with distant metastasis are not suitable candidates for conventional therapy and exhibit a five-year survival rate of <10% (Brenner et al., 2014; Cao et al.,
Therefore, it is urgent to make clear the metastasis, progress mechanism of CRC and establish a new therapeutic target for CRC patients with distant metastases. Recently, more and more studies have demonstrated that dysregulation of lncRNAs could significantly influence CRC cancer origination and progression including cell metastasis. For example, the abnormal expression of long non-coding RNA UPAT (UHRF1 Protein Associated Transcript) promotes CRC proliferation and tumorigenicity by inhibiting degradation of UHRF1 (Brenner et al., 2014; Cao et al., 2015). LncRNA HOTAIR expression is associated with polycomb-dependent chromatin modification and is always a factor for poor prognosis and metastasis in CRCs (Kogo et al., 2011). Moreover, it has been reported that the colon cancer–associated transcript 2 acts as an lncRNA, namely CCAT2, to regulate cell proliferation, metastasis, and chromosomal instability in microsatellite-stable CRC (Ling et al., 2013). In our current study, we found that HOTTIP has an oncogenic function in CRC though promoting migration and invasion of cells, which is consistent with its higher expression levels in CRC tissues compared with normal tissues.

Similar to previous studies and reported by Ren (Ling et al., 2013), overexpression of HOTTIP is an unfavorable factor in CRC patients because a higher expression level of HOTTIP was closely related to patients with larger tumor size, advanced pathological stage, or distant metastasis. In a previous study, Lian et al demonstrated that HOTTIP could promote CRC growth and inhibit cell apoptosis, partially via downregulation of p21 expression (Lian et al., 2016). Given this, we hypothesize that HOTTIP may play a role in CRC metastasis. Considering the endogenous expression level of HOTTIP in CRC cells, we further explored the biological function and underlying mechanism using SW480 and Caco2 as cell model. Sequentially, CRC cell metastasis was assessed by MTT assay, colony formation, migration assay, and invasion assay. The results indicated that silencing HOTTIP could inhibit CRC cells growth and metastasis in vivo and in vitro, while overexpression of HOTTIP had the opposite effect. Next, to identify the downstream targets of HOTTIP, we first assessed the
expression of the metastasis-associated and EMT-associated genes via qRT-PCR. Among these analyzed genes, we found that DKK1 was the most significantly altered gene after HOTTIP knockdown or overexpression, and this result was confirmed by western blot.

DKK1 was first identified in Xenopus and functioned as a potent antagonist of Wnt/β-catenin signaling (Glinka et al., 1998). Although some studies have reported that DKK1 levels are elevated in a wide variety of cancers, several reports suggest that DKK1 may have tumor-suppressing functions, and downregulation or loss of DKK1 expression has been well documented in a number of clinical studies involving breast cancer, renal cell carcinoma, melanoma, and colon cancer (Gonzalez-Sancho et al., 2005; Hirata et al., 2011; Kuphal et al., 2006; Menezes et al., 2012; Mikheev et al., 2008). Recently, several studies indicated that DKK1 may function as a tumor suppressor which is involved in a cancer-related ncRNA regulated network. For example, IncRNA NBAT1 (neuroblastoma associated transcript 1) inhibits migration and invasion of breast cancer cells by activating DKK1 expression (Hu et al., 2015). miR-493 modulated the proliferation, invasion, and chemosensitivity of gastric cancer cells via suppressing DKK1 expression (Jia et al., 2016). Interestingly, our data showed that DKK1 was negatively regulated by the oncogenic function of HOTTIP at the mRNA and protein levels in CRC cells.

To further investigate whether DKK1 was involved in the HOTTIP-induced CRC cell migration and invasion, we carried out rescue experiments. Results indicated that when co-transfected with HOTTIP and DKK1, siRNAs could partially rescue si-HOTTIP inhibited metastasis in SW480 cells. Taken together, these findings suggest that HOTTIP promotes CRC cell migration and invasion partly through downregulation of DKK1 expression. In addition, further analyses are needed to better understand the mechanisms between HOTTIP and DKK1. Other possible downstream targets and mechanisms by which HOTTIP participates in CRC cell metastasis remain to be further investigated.
In summary, our study reveals a unique role of HOTTIP in CRC – that is, that HOTTIP mediated DKK1 downregulation confers metastasis and invasion in CRC cells. Moreover, our data also identified that patients exhibiting high HOTTIP expression have higher metastasis potential and poor clinical outcomes.

Additional Files

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by the Science and Technology Program (Industrial Technology Innovation Special Projects) of Suzhou, China(no.SS201755), the Program of Science and Education to Promote Health of Wujiang District, Suzhou, China(no.WWK201617)

References:


long noncoding RNA HOXA transcript at the distal tip promotes colorectal cancer growth partially via silencing of p21 expression. Tumour Biol. 37, 7431-7440.


**Table 1.** The clinicopathological factors of CRC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Expression level of HOTTIP</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n=26)</td>
<td>High(n=27)</td>
</tr>
<tr>
<td><strong>Age(years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>&gt;50</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td><strong>Tumor size(cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤5</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>&gt;5</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td><strong>Histologic differentiation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Poorly</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td><strong>TNM Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>III/IV</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Negative</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td><strong>Distant metastasis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>15</td>
</tr>
</tbody>
</table>

*Chi-square test. * P < 0.05

**Figure. 1 Increased HOTTIP expression in CRC tissues and its clinical significance. a.** Relative expression of HOTTIP in CRC tissues (N = 53) compared with the corresponding adjacent normal tissues (N = 53). HOTTIP expression was examined by qRT-PCR and normalized to GAPDH expression. The results are presented as the fold-change in tumor tissues relative to normal tissues. **b-d.** Higher HOTTIP was positively correlated with a larger tumor size, a higher pathological stage, and distant metastasis. Bars: s.d, * P < 0.05, ** P < 0.01, from three independent experiments

**Figure. 2 The expression levels of HOTTIP in CRC cells. a.** qRT-PCR analysis of HOTTIP expression in CRC cells. **b,c.** Detection of HOTTIP expression level in CRC cells after silencing of HOTTIP or overexpression of HOTTIP by qRT-PCR. Bars: s.d, * P < 0.05, ** P < 0.01, from three independent experiments

**Figure. 3 Knockdown of HOTTIP expression inhibited metastasis of CRC cell. a,b.** MTT assays and colony forming assays were used to determine the cell viability of SW480 cells transfected with siRNAs against HOTTIP. **c.** Wound healing assays were used to investigate the migratory ability of SW480 cells. **d,e.** Transwell assays
were used to investigate the changes in the migratory and invasive abilities of SW480 cells. Bars: s.d, *P<0.05, **P<0.01, from three independent experiments

Figure. 4 Enhanced metastasis of CRC cell with overexpressed HOTTIP. a,b. MTT assays and colony forming assays were used to determine the cell viability of SW480 cells transfected with pcDNA-HOTTIP. c. Wound healing assays were used to investigate the migratory ability of SW480 cells. d,e. Transwell assays were used to investigate the changes in the migratory and invasive abilities of SW480 cells. Bars: s.d, *P<0.05, **P<0.01, from three independent experiments

Figure. 5 Silencing HOTTIP inhibits tumor metastasis of CRC in vivo. a. SW480 cells transfected with sh-HOTTIP and empty vectors were separately injected into the tail veins of xenograft mouse. Visualization of the entire lung, and hematoxylin and eosin (HE)-stained lung sections. b. qRT-PCR analysis of HOTTIP expression in xenograft tumor tissues. c. Lungs were harvested from the mouse in each experimental group, and the numbers of tumor nodules visible on lung surfaces were counted. Bars: s.d, *P<0.05, **P<0.01

Figure. 6 Regulation of DKK1 expression by HOTTIP. a. Heat map showing expression levels of 10 different expressed transcripts in SW480 cells with HOTTIP knockdown. b,c. Expression of cell adhesion molecules (E-cadherin, N-cadherin, vimentin, DKK1, EMP1, and FN1) as detected using qRT-PCR after HOTTIP was blocked or overexpressed in SW480 cells. d. The expression of DKK1 protein in SW480 cells was analyzed by western blotting. Bars: s.d, *P<0.05, **P<0.01, from three independent experiments

Figure. 7 HOTTIP promotes metastasis by repressing DKK1 expression in CRC cell. a. Detection of DKK1 expression level in SW480 cells after silencing of DKK1. b. Colony forming assays were used to determine the cell viability for si-HOTTIP and si-DKK1 co-transfected SW480 cells. c,d. Transwell assays were used to investigate
the changes in the migratory and invasive abilities for si-HOTTIP and si-DKK1 co-transfected SW480 cells. Bars: s.d,*P<0.05,**P<0.01, from three independent experiments

(Jia et al., 2016)