

# lncRNA HOTAIR Contributes to 5FU Resistance through Suppressing miR-218 and Activating NF- $\kappa$ B/TG Signaling in Colorectal Cancer

Peilong Li,<sup>1</sup> Xin Zhang,<sup>2</sup> Lili Wang,<sup>2</sup> Lutao Du,<sup>2</sup> Yongmei Yang,<sup>2</sup> Tong Liu,<sup>1</sup> Chen Li,<sup>1</sup> and Chuanxin Wang<sup>1</sup>

<sup>1</sup>Department of Clinical Laboratory, The Second Hospital of Shandong University, Jinan, 250033 Shandong Province, China; <sup>2</sup>Department of Clinical Laboratory, Qilu Hospital, Shandong University, Jinan, 250012 Shandong Province, China

**One major reason for the failure of advanced colorectal cancer (CRC) treatment is the occurrence of chemoresistance to fluoropyrimidine (FU)-based chemotherapy. Long non-coding RNA HOTAIR has been considered as a pro-oncogene in multiple cancers. However, the precise functional mechanism of HOTAIR in chemoresistance is not well known. In this study, we investigated the biological and clinical role of HOTAIR in 5FU resistance in CRC. Our results showed that HOTAIR negatively regulated miR-218 expression in CRC through an EZH2-targeting miR-218-2 promoter regulatory axis. HOTAIR knockdown dramatically inhibited cell viability and induced G1-phase arrest by promoting miR-218 expression. VOPPI was shown to be a functional target of miR-218, and the main downstream signaling, NF- $\kappa$ B, was inactivated by HOTAIR through the suppression of miR-218 expression. Additionally, HOTAIR knockdown partially reversed 5FU resistance through promoting miR-218 and inactivating NF- $\kappa$ B signaling. Furthermore, HOTAIR restrained 5FU-induced cytotoxicity on CRC cells through promotion of thymidylate synthase expression. More importantly, high HOTAIR expression was associated with poor response to 5FU treatment. In conclusion, we demonstrated that HOTAIR contributes to 5FU resistance through suppressing miR-218 and activating NF- $\kappa$ B signaling in CRC. Thus, HOTAIR may serve as a promising therapeutic target for CRC patients.**

## INTRODUCTION

Colorectal cancer (CRC) is among the leading causes of mortality and morbidity throughout the world. It is the third most common cancer and the fourth most common cause of oncological deaths worldwide.<sup>1</sup> Nearly a quarter of patients with CRC experience metastatic disease and receive standard chemotherapy regimens. However, most of these patients eventually become chemoresistant and die of their diseases.<sup>2</sup> Therefore, it is essential to understand mechanisms of chemoresistance as a first step in developing approaches to prevent or reverse chemoresistance in patients who receive systemic therapy for metastatic CRC.

5-Fluorouracil (5FU) is one of the mainstays of chemotherapeutic regimens for metastatic CRC. It works by multiple mechanisms to

create fluorinated nucleotides that are incorporated into DNA in place of thymidine, thus inhibiting DNA replication and causing cell death.<sup>3</sup> Its use in combination with oxaliplatin and leucovorin (FOLFOX) for metastatic CRC has led to response rates >50% and median survival approaching 2 years.<sup>4</sup> However, it is far from being the perfect treatment. Nearly half of metastatic CRC patients are resistant to 5FU-based chemotherapy.<sup>5,6</sup> Although resistance mechanisms have been extensively studied for this agent, therapies to target resistance pathways have yet to be identified.

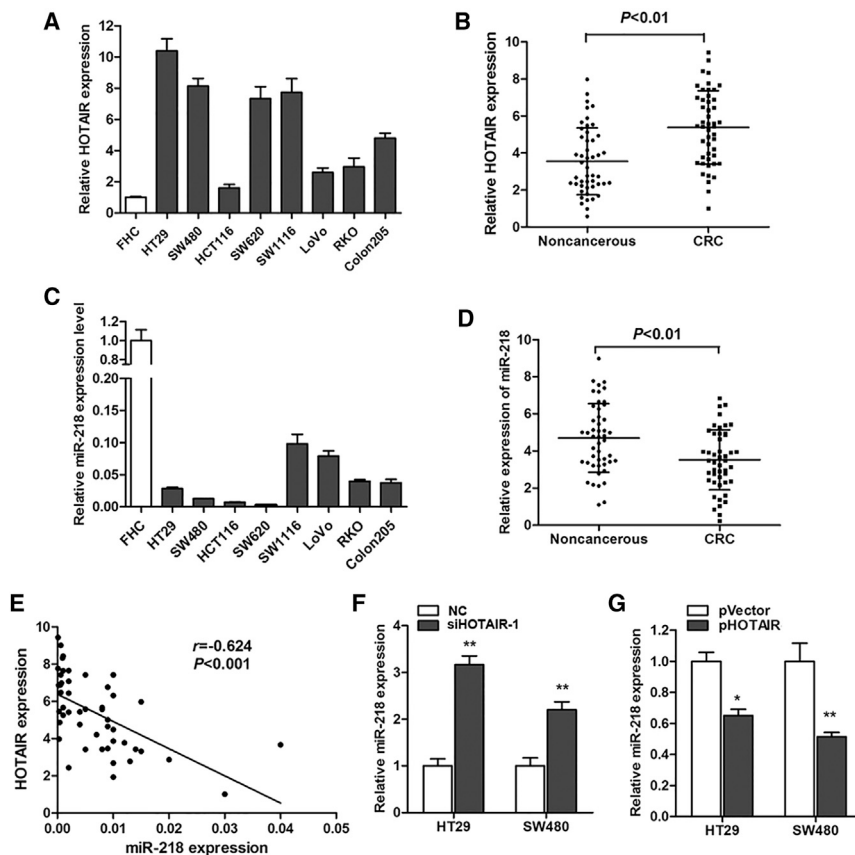
Long non-coding RNAs (lncRNAs), extensively transcribed from the mammalian genome, have gained widespread attention in recent years. They serve as important and powerful regulators of various biological activities and play critical roles in the progression of a variety of diseases, including cancer.<sup>7,8</sup> lncRNA HOTAIR (Hox transcript antisense intergenic RNA) is a 2,158 bp lncRNA located in the HOXC gene cluster (12q13.13). HOTAIR trimethylates histone H3 lysine-27 (H3K27me3) of the targeted gene locus with the polycomb-repressive complex 2 (PRC2), which is composed of EZH2, SUZ12, and EED and inhibits gene expression.<sup>9</sup> Aberrant HOTAIR expression is positively correlated with progression as well as poor survival and prognosis of various types of cancers, such as breast,<sup>10</sup> gastric,<sup>11</sup> and CRC.<sup>12</sup> However, the potential involvement of HOTAIR in the chemoresistance of CRC is not well known.

The crosstalk between lncRNAs and microRNAs (miRNAs) has recently been reported to contribute to the pathogenesis of diseases, including cancer.<sup>13</sup> As a broadly conserved miRNA, miR-218 is considered to be a tumor suppressor gene in various carcinomas, such as bladder cancer,<sup>14</sup> gastric cancer,<sup>15</sup> non-small-cell lung cancer,<sup>16</sup> and CRC.<sup>17</sup> Downregulation of miR-218 is a consequence of the epigenetic silencing of its host genes, SLIT2 and SLIT3

Received 11 May 2017; accepted 6 July 2017;  
<http://dx.doi.org/10.1016/j.omtn.2017.07.007>

**Correspondence:** Chuanxin Wang, Department of Clinical Laboratory, The Second Hospital of Shandong University, No. 247 Beiyuan Street, Jinan, 250033 Shandong Province, China.

**E-mail:** [cxwang@sdu.edu.cn](mailto:cxwang@sdu.edu.cn)



**Figure 1. HOTAIR Negatively Regulated miR-218 Expression in CRC Cells**

(A and B) qRT-PCR showed that HOTAIR was upregulated in CRC cell lines (A) and primary CRC tissues (B). (C and D) miR-218 was downregulated in CRC cell lines (C) and primary CRC tissues (D). (E) Spearman test indicated a significantly negative correlation between HOTAIR and miR-218 expression in primary CRC tissues. (F and G) miR-218 was upregulated by siHOTAIR-1 (F), whereas it was downregulated by pHOTAIR (G) in HT29 and SW480 cells. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .

specimens (Figures 1C and 1D). Moreover, the Spearman test indicated a significantly negative correlation between HOTAIR and miR-218 expression in primary CRC tissues (Figure 1E). To investigate the potential role of HOTAIR in CRC cells, we downregulated its expression by transfection of siHOTAIR. The results showed that HOTAIR expression was significantly suppressed by siHOTAIR-1, siHOTAIR-2, and siHOTAIR-3 (Figure S1A). siHOTAIR-1 was selected for use in the following experiments. After knockdown of HOTAIR, miR-218 was significantly increased in HT29 and SW480 cells (Figure 1F). On the other hand, miR-218 was downregulated in the aforementioned cell lines with HOTAIR overexpression (Figures 1G and S1B). Thus,

we concluded that HOTAIR negatively regulated miR-218 in CRC cells.

#### HOTAIR Was Associated with EZH2, which Subsequently Suppressed miR-218 Expression

HOTAIR exerts its oncogenic role by functioning as a bridge to recruit PRC2, thereby leading to gene methylation and chromatin modifications.<sup>9</sup> EZH2, a critical component of PRC2, is frequently upregulated in CRC and possesses oncogenic properties in tumorigenesis.<sup>22,23</sup> In our study, EZH2 was upregulated in CRC tissues (Figure S2A). The small interfering RNAs (siRNAs) against EZH2 were designed, and its expression was decreased by siEZH2-1 and siEZH2-2 at both the RNA and protein levels (Figure S2B). Moreover, the cell viability was suppressed by siEZH2-1 (Figure S2C). To investigate whether HOTAIR is associated with EZH2 in CRC, an RNA immunoprecipitation (RIP) assay was performed, and the results showed a significant enrichment of HOTAIR with EZH2 antibody compared with the non-specific IgG (immunoglobulin G) antibody in both HT29 and SW480 cells (Figure 2A).

Downregulation of miR-218 is a consequence of the epigenetic silencing of their host genes, SLIT2 and SLIT3 promoters.<sup>18</sup> Previous studies have reported that SLIT3, consistent with miR-218-2 precursor, was silenced in pancreatic cancer and thyroid cancer.<sup>19,24</sup>

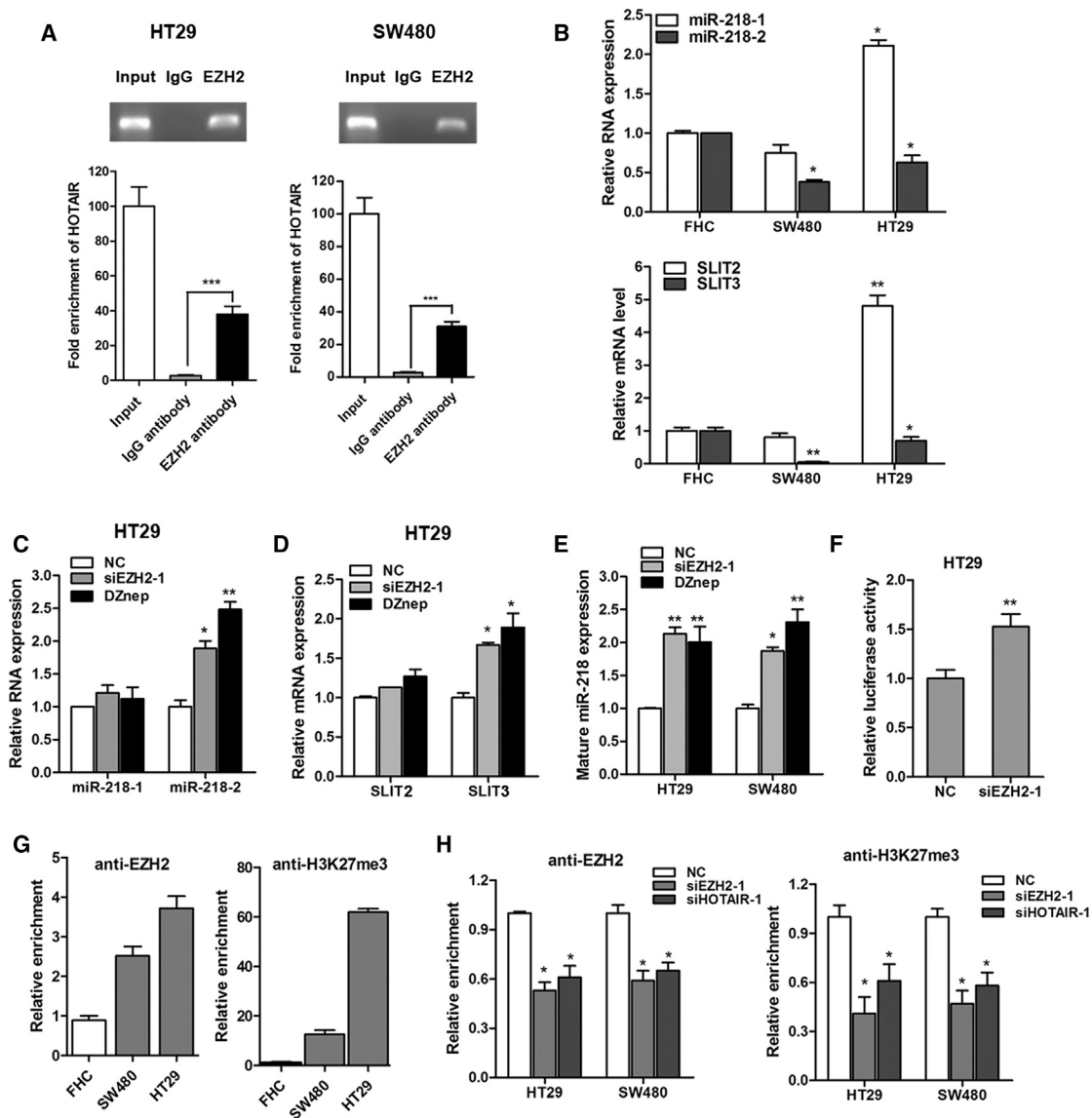
promoters.<sup>18</sup> A previous study demonstrated that EZH2 silenced miR-218 by inducing the formation of heterochromatin in pancreatic cancer,<sup>19</sup> which indicated that the research on epigenetic regulation of miR-218 and HOTAIR might be meaningful.

In our previous study, we identified the role of lncRNA MALAT1 and miR-218 in CRC chemoresistance.<sup>20,21</sup> On this basis, our further investigation revealed that the suppression of miR-218 might be mediated by HOTAIR in an EZH2-dependent manner. The enforced HOTAIR expression promoted CRC cell viability and chemoresistance through a miR-218/NF (nuclear factor)- $\kappa$ B pathway. Moreover, HOTAIR expression was significantly increased and associated with poor survival rate in CRC patients who experienced chemoresistance to 5FU-based treatments.

## RESULTS

### HOTAIR Negatively Regulates miR-218 Expression in CRC Cells

First, we sought to determine the interaction between HOTAIR and miR-218. HOTAIR was commonly considered as an oncogene in several cancers. As expected, our results showed that it was significantly increased in most CRC cell lines (Figure 1A). We also found that HOTAIR was increased in 48 primary CRC tissues, compared with adjacent tissues (Figure 1B). Consistent with our previous research, miR-218 was sufficiently downregulated in CRC cells and



**Figure 2. HOTAIR Suppressed miR-218 Expression through an EZH2-miR-218-2 Promoter Regulatory Axis**

(A) HOTAIR expression was significantly enriched by EZH2 antibodies in the immunoprecipitation assay. (B) SLIT3 and miR-218-2 were significantly downregulated in HT29 and SW480 cells, while SLIT2 and miR-218-1 were upregulated in HT29 cells, compared to normal FHC cells. (C and D) miR-218-2 (C) and its host gene, SLIT3 (D), were significantly upregulated by siEZH2-1 and DZnep (3-deazaneplanocin), an EZH2 inhibitor, while miR-218-1 and SLIT2 were less responsive in HT29 cells. (E) The total miR-218 expression was also upregulated by siEZH2-1 in CRC cells. (F) siEZH2-1 promoted the luciferase activity of the miR-218-2-Luc in HT29 cells. (G) ChIP analysis using anti-EZH2 and anti-H3K27me3 was conducted on the miR-218-2 promoter. A significant enrichment was found by anti-EZH2 and anti-H3K27me3 in CRC cells but not in FHC cells. (H) ChIP analysis also showed that the binding levels of EZH2 and H3K27me3 on the miR-218-2 promoter was significantly decreased by siEZH2-1 or siHOTAIR-1 in CRC cells. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

In our study, we found that SLIT3 and miR-218-2 were significantly downregulated in both CRC cells, while SLIT2 and miR-218-1 were upregulated in HT29 cells (Figure 2B). Moreover, SLIT3 was also found to be downregulated, whereas SLIT2 remained unchanged in CRC tissues (Figure S2D). This suggests that the downregulation of mature miR-218 in CRC may be a consequence of epigenetic silencing of SLIT3 promoter. As expected, miR-218-2 precursor and SLIT3

gene were significantly increased after knockdown of EZH2 with si-EZH2-1 and DZnep, an EZH2 inhibitor, while miR-218-1 and SLIT2 were less responsive in HT29 cells (Figures 2C and 2D). Additionally, mature miR-218 expression was also promoted by si-EZH2-1 and DZnep in HT29 and SW480 cells (Figure 2E). Further, the luciferase assay was also performed in HT29 cells. A 2,000-bp sequence of miR-218-2 promoter was cloned into PGL3 luciferase

reporter (miR-218-2-Luc), and the activity of the miR-218-2 promoter was then validated in HT29 cells (Figure S2E). As expected, the knockdown of EZH2 generated a higher luciferase activity of miR-218-2-Luc compared with control (Figure 2F). These suggest a unique function of EZH2 on miR-218-2 suppression in CRC. To verify the definite regulatory role of HOTAIR-EZH2 complex on miR-218, we used chromatin immunoprecipitation (ChIP) analysis to determine the effect of HOTAIR-EZH2 complex on histone modification in the miR-218-2 promoter with an anti-EZH2 antibody and an anti-H3K27me3 antibody in CRC cells. EZH2 and H3K27me3 were frequently found at the miR-218-2 promoter of CRC cells but not in FHC (human fetal normal colonic) cells (Figure 2G). The binding level of EZH2 and H3K27me3 was significantly decreased by siEZH2-1 or siHOTAIR-1 in CRC cells (Figure 2H), suggesting that the interaction of EZH2 and H3K27me3 on the miR-218-2 promoter is specifically mediated by HOTAIR. In summary, we proved that HOTAIR suppressed miR-218 expression through epigenetically silencing SLIT3 by recruiting EZH2 in CRC.

#### HOTAIR Promoted Cell Viability by Downregulating miR-218 Expression

After transfection with siHOTAIR or pHOTAIR, cell viability was evaluated, and the results showed that siHOTAIR suppressed cell viability, while pHOTAIR promoted cell survival in CRC cells (Figure 3A). We then investigated the effect of miR-218 on CRC cell viability. As shown in Figure 3B, miR-218 suppressed cell viability, while anti-miR-218 induced a proliferative effect on cell viability. Cell-cycle analysis indicated that siHOTAIR or ectopic miR-218 induced an increase in the percentage of cells in the G1 phase (Figure 3C). Next, the capacity of colony formation was assessed, and the results showed that siHOTAIR-transfected cells displayed highly suppressed colony formation (Figure 3D).

To evaluate the antagonistic effect of HOTAIR and miR-218 on cell viability, miR-218 was transfected into the pHOTAIR stably infected HT29 and SW480 cells. As shown in Figure 3E, miR-218 reversed the proliferative effect induced by HOTAIR overexpression. On the other hand, suppression of miR-218 significantly abrogated the growth inhibition induced by HOTAIR knockdown. Furthermore, the cell proliferation marker Ki-67 was detected by immunofluorescence assay. Anti-miR-218 partially rescued the siHOTAIR-induced suppression of Ki-67 expression in HT29 cells (Figure 3F), which was also validated by western blot (Figure S3). These data suggest that HOTAIR promotes CRC progression through suppressing miR-218 expression.

#### VOPPI Was Identified as a Direct Target of miR-218 in CRC Cells

VOPPI was predicted to be the functional target of miR-218 by several bioinformatics programs, including TargetScan and miRanda (Figure S4). qRT-PCR showed that VOPPI was upregulated in CRC tissues (Figure 4A), and a significantly negative correlation was found between VOPPI and miR-218 expression in 48 CRC tissues (Figure 4B). Overexpression of miR-218 decreased both VOPPI mRNA and protein expression in CRC cells (Figure 4C). Additionally, the

luciferase activity assay showed that miR-218 dramatically inhibited the firefly luciferase activity of the mutant VOPPI-3' UTR, but not that of the mutant VOPPI-3' UTR, in both HT29 and SW480 cells (Figure 4D). More importantly, VOPPI was significantly upregulated by pHOTAIR at mRNA and protein levels (Figure 4E). These results indicate that HOTAIR may regulate CRC progression, at least partially, through the suppression of miR-218 and the activation of VOPPI expression.

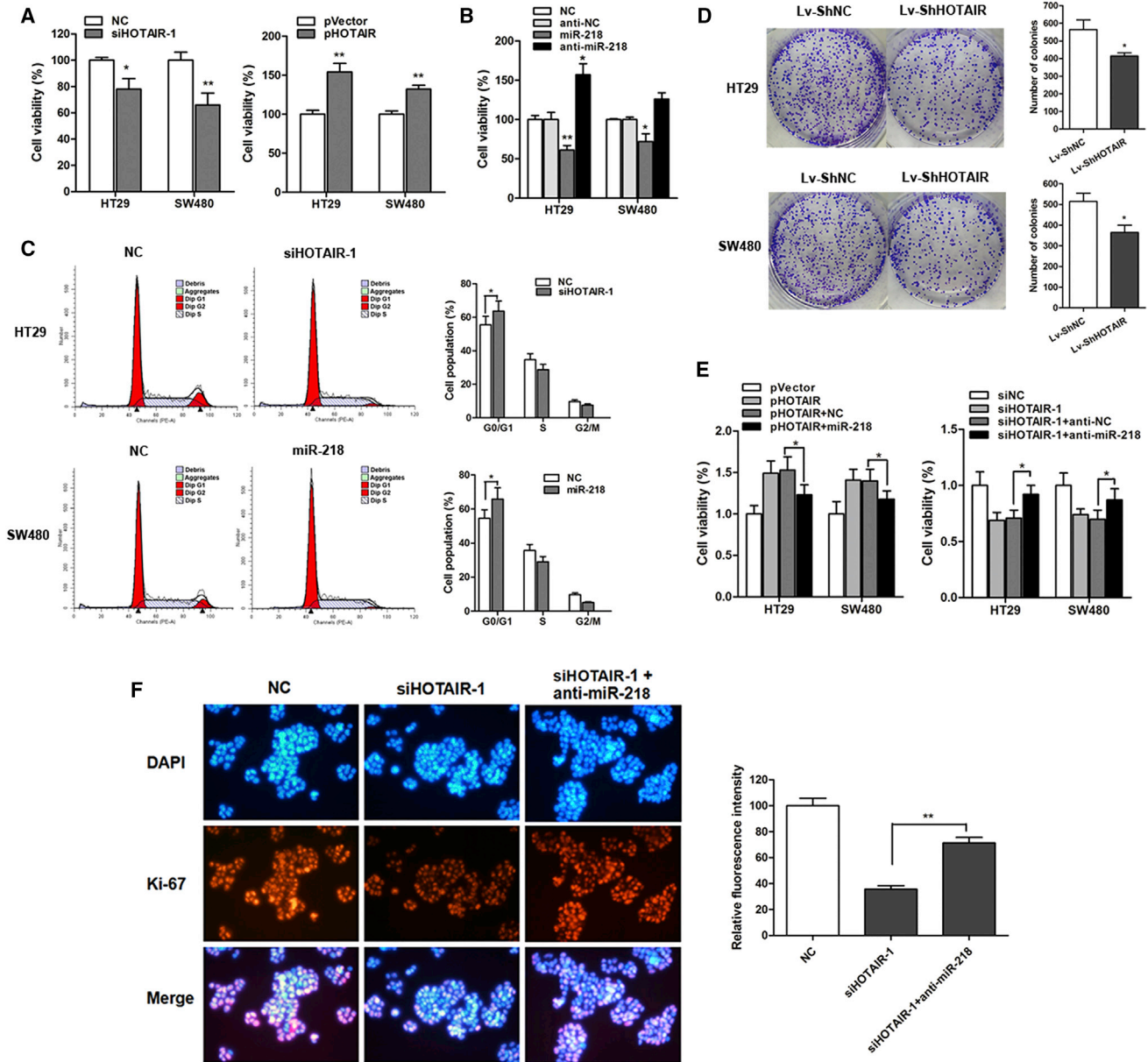
#### NF- $\kappa$ B Signaling Was Activated by HOTAIR through the Suppression of miR-218 Expression

After revealing the interaction between miR-218 and VOPPI, we sought to elucidate whether miR-218 suppressed cell proliferation by reducing VOPPI expression. First, we silenced VOPPI expression using its specific siRNAs (siVOPPI), and the results revealed that it was significantly suppressed at both the mRNA and protein levels in HT29 cells (Figure S5A). Then, the siVOPPI-1 was used, and it significantly attenuated cell growth and induced G1-phase arrest in HT29 cells (Figures 5A and 5B). Moreover, VOPPI knockdown dramatically suppressed the enhanced cell growth induced by anti-miR-218 (Figure 5C). We next determined whether VOPPI overexpression could reverse the suppressive effect of miR-218 in CRC cells. An expression vector PcDNA-VOPPI, which encoded the full-length coding sequence of VOPPI, was transfected into HT29 cells (Figure S5B). Intriguingly, reinforced expression of VOPPI enhanced cell growth (Figure 5D). More importantly, the reinforced expression of VOPPI dramatically abrogated miR-218-induced cell proliferation inhibition (Figure 5E).

VOPPI has been well established as a key transcriptional regulator of cell survival and the cell cycle through the activation of the NF- $\kappa$ B signaling pathway,<sup>25,26</sup> and our data validated that VOPPI knockdown significantly inactivated the NF- $\kappa$ B signaling pathway, including the suppression of pathways involved in cell survival (p65-NF- $\kappa$ B, pAkt, and pERK) and the cell cycle (E2F-1 and TS) (Figure 5F). Therefore, we questioned whether endogenous NF- $\kappa$ B pathway activation was affected by ectopic expression of HOTAIR and miR-218. As expected, the expression of p65-NF- $\kappa$ B, pAkt, pERK, E2F-1, and TS was significantly decreased after knockdown of HOTAIR, while they were upregulated by transfection of pHOTAIR, indicating the positive regulation of HOTAIR on NF- $\kappa$ B signaling (Figure 5G). On the other hand, the NF- $\kappa$ B signaling pathway was inactivated by miR-218 overexpression and activated by inhibition of miR-218 (Figure 5H). This indicated that NF- $\kappa$ B signaling was activated by HOTAIR through suppressing miR-218 expression in CRC.

#### HOTAIR Knockdown Partially Reversed 5FU Resistance through the Promotion of miR-218 and the Inactivation of NF- $\kappa$ B/TS Signaling

We aimed to investigate whether the HOTAIR-miR-218 pathway participates in the 5FU resistance in CRC. The CRC cell line HT29 that had acquired resistance to 5FU at the clinically relevant concentration of 2  $\mu$ g/mL (HT29 5FU-R [5FU-resistant] cells) was built and validated (Figures S6A-S6E). Then, the HOTAIR and miR-218

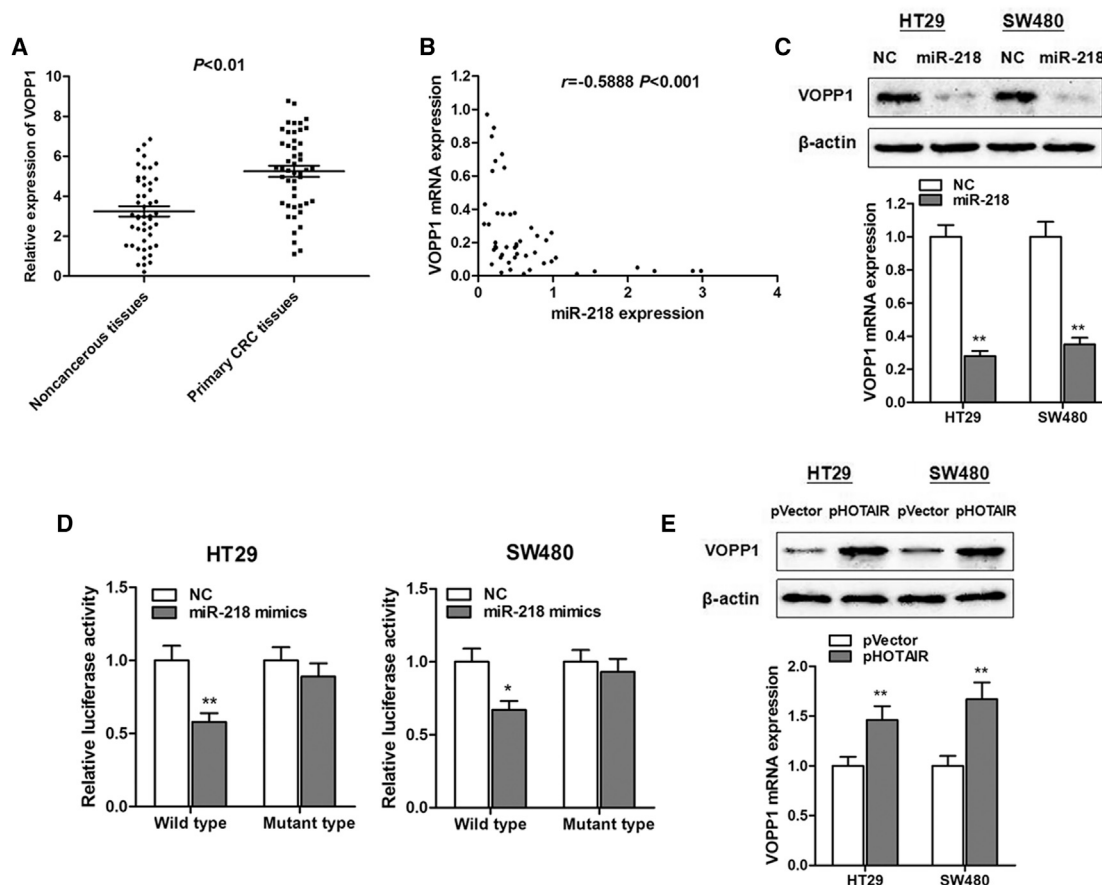


**Figure 3. HOTAIR Promoted Cell Viability by Downregulating miR-218 Expression**

(A) CCK8 assay showed that siHOTAIR suppressed cell viability (left panel), while pHOTAIR promoted cell survival (right panel) in CRC cells. (B) miR-218 suppressed cell proliferation, whereas anti-miR-218 promoted cell proliferation in CRC cells. (C) The cell-cycle analysis indicated that HOTAIR knockdown (upper panels) or miR-218 overexpression (lower panels) induced an increase in the percentage of cells in the G1 phase. (D) The representative pictures and statistical analyses of the colony formation assay of LV-ShHOTAIR-infected CRC cells. (E) The co-transfection assay showed that miR-218 reversed the proliferative effect induced by HOTAIR overexpression (left panel), while suppression of miR-218 significantly abrogated growth inhibition induced by HOTAIR knockdown (right panel). (F) The immunofluorescence of Ki-67 (stained sections followed by counterstaining with DAPI) showed that anti-miR-218 partially rescued the siHOTAIR-induced suppression of Ki-67 expression in HT29 cells. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .

expression were detected, and the results showed that HOTAIR was upregulated while miR-218 was downregulated in 5FU-R cells compared to parental cells (Figure 6A). To evaluate their potential function in 5FU resistance, siHOTAIR-1 was transfected into HT29 5FU-R cells. The cells became sensitive to 5FU after HOTAIR

knockdown, with only 60% of viable cells remaining after exposure to 2  $\mu\text{g}/\text{mL}$  5FU for 72 hr, while cells transfected with blank vectors remained resistant to 5FU, with 91% of viable cells remaining (Figure 6B). Similarly, miR-218 mimics promoted 5FU-induced cell cytotoxicity, compared to negative control duplex (NC) (64% versus 93%,



**Figure 4. VOPP1 Was Identified as a Direct Target of miR-218 in CRC Cells**

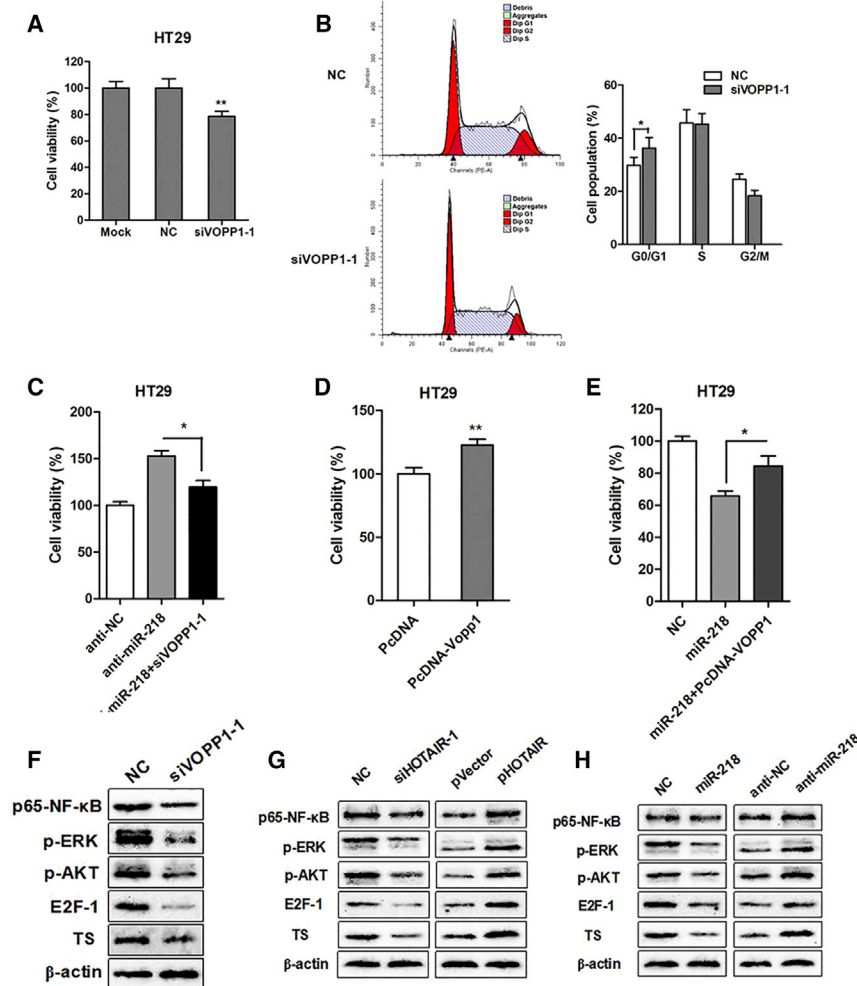
(A) VOPP1 was upregulated in CRC tissues, compared to paired non-cancerous tissues. (B) The Spearman test indicated a significantly negative correlation between VOPP1 and miR-218 expression in 48 CRC tissues. (C) VOPP1 mRNA (lower panel) and protein (upper panel) expression levels were significantly suppressed by miR-218 in CRC cells. (D) Luciferase activity assay showed that miR-218 dramatically inhibited the firefly luciferase activity of the mutant VOPP1-3' UTR, but not that of the mutant VOPP1-3' UTR, in both HT29 and SW480 cells. (E) VOPP1 mRNA (lower panel) and protein (upper panel) expression were suppressed by siHOTAIR-1, whereas they were upregulated by pHOTAIR. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .

respectively) (Figure 6C). More importantly, anti-miR-218 partially abrogated the enhanced susceptibility to 5FU induced by HOTAIR knockdown (Figure 6D). These results indicate that HOTAIR inhibition may contribute to the 5FU-induced cytotoxicity through the promotion of miR-218 in CRC cells.

Subsequently, we determined whether the VOPP1/NF- $\kappa$ B pathway was involved in 5FU resistance. As expected, VOPP1 was upregulated, and the NF- $\kappa$ B pathway was activated in HT29 5FU-R cells, compared with parental cells (Figure 6E). However, NF- $\kappa$ B signaling was inactivated after transfection with siHOTAIR-1 or miR-218 in HT29 5FU-R cells (Figure 6F). VOPP1 knockdown also inactivated NF- $\kappa$ B signaling in 5FU-R cells (Figure 6G), and VOPP1 overexpression partially reversed the miR-218-induced enhanced susceptibility to 5FU in the HT29 5FU-R subline (Figure 6H). Thus, we revealed that HOTAIR knockdown partially reversed 5FU resistance through promoting miR-218 and inactivating NF- $\kappa$ B signaling.

#### HOTAIR Restrained 5FU-Induced Cytotoxicity on CRC Cells through the Suppression of miR-218 and the Promotion of TS

After being transfected with pHOTAIR, HT29 cells were incubated without 5FU or with a concentration gradient of 1, 1.5, 2, 3, and 6  $\mu$ g/mL 5FU for 72 hr. Then, a dose-effect curve was constructed based on the viability of cells. The graph demonstrated that pHOTAIR transfection decreased the 5FU-induced cell cytotoxicity, compared with the negative vector; however, co-transfection with miR-218 mimics partially reversed this effect in both HT29 and SW480 cells (Figures 6I and 6J). Furthermore, the median inhibitory concentration ( $IC_{50}$ ) of 5FU on CRC cells was detected, and the results indicated that the  $IC_{50}$  value of 5FU was 3.2  $\mu$ g/mL and 2.3  $\mu$ g/mL in non-transfected HT29 and SW480 cells, respectively. However, the 5FU  $IC_{50}$  values increased to 5.8  $\mu$ g/mL and 4.8  $\mu$ g/mL when HOTAIR was overexpressed with pHOTAIR in HT29 and SW480 cells, respectively, while these values in HT29 cells and SW480 cells co-transfected with HOTAIR and miR-218 were 4.2  $\mu$ g/mL and 3.7  $\mu$ g/mL, respectively. More importantly,



**Figure 5. NF- $\kappa$ B Pathway Signaling Was Activated by HOTAIR through the Suppression of miR-218 Expression**

(A) Knockdown of VOPP1 suppressed the cell viability in HT29 cells. (B) siVOPP1-1 induced G1-phase arrest in HT29 cells. (C) siVOPP1-1 dramatically suppressed the enhanced cell proliferation induced by anti-miR-218 in HT29 cells. (D and E) Reinforced expression of VOPP1 could enhance cell growth (D) and further abrogate miR-218-induced cell proliferation inhibition (E) in HT29 cells. (F) siVOPP1-1 inactivated NF- $\kappa$ B signaling, including the suppression of pathways involved in cell survival (p65-NF- $\kappa$ B, pAkt, pERK) and the cell cycle (E2F-1 and TS). (G) The NF- $\kappa$ B signaling was significantly suppressed by HOTAIR knockdown, while it was activated by transfection of pHOTAIR. (H) miR-218 induced the inactivation of NF- $\kappa$ B signaling, whereas anti-miR218 induced the activation of the NF- $\kappa$ B signaling pathway. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .

area under the curve (AUC) and the diagnostic sensitivity and specificity reached 0.716, 81.58%, and 55.26% with the established cutoff value, respectively. The proportion of patients not responding to chemotherapy was significantly higher in the high-HOTAIR-expression group than in the low-expression group (Figure 7B). Clinicopathologic factors were then analyzed, and the high-HOTAIR-expression group showed a less differentiated histology, larger tumor size, and enhanced distant metastasis, compared to the low-HOTAIR-expression group (Table 1). Additionally, 80 of the 152 patients were successfully exposed to a Kaplan-Meier survival analysis, and the results indicated

immunofluorescence and western blot analyses showed that TS protein was suppressed in HT29 cells after the treatment of 2  $\mu$ g/mL 5FU for 72 hr. However, the suppression level of TS was relieved when the cells were transfected with pHOTAIR or anti-miR-218 before 5FU treatment (Figures 6K and S6F). To conclude, HOTAIR exerts its chemoresistant role through targeting TS enzyme.

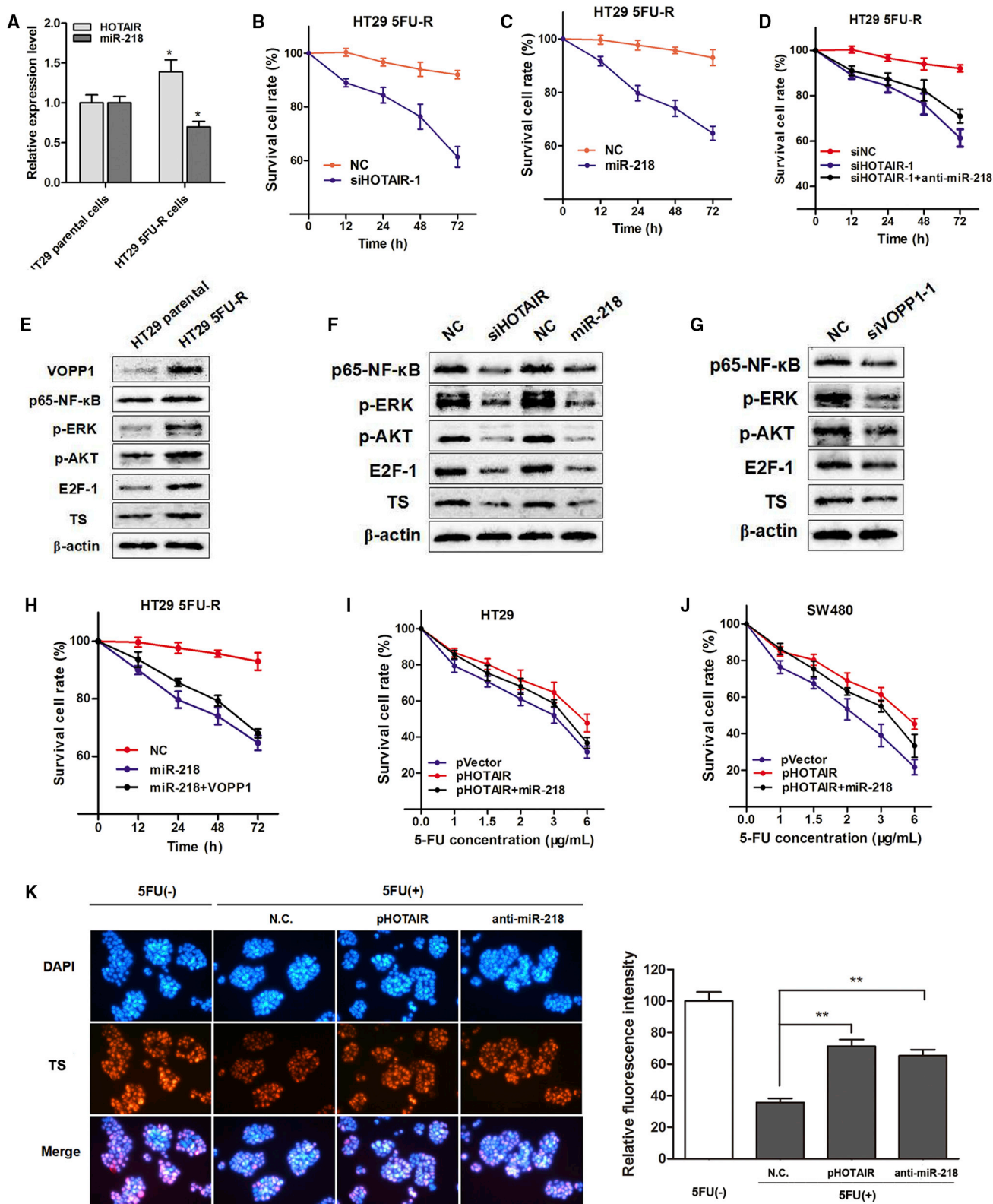
#### A High HOTAIR Expression Level Is Associated with Poor Response to First-Line 5FU Treatment in CRC Patients

We attempted to explore the clinical value of HOTAIR in 152 CRC patients receiving 5FU based treatment. qRT-PCR analysis showed that the HOTAIR expression level was much higher in CRC tissues from patients who did not respond to 5FU treatment than those from patients who experienced response to chemotherapy (Figure 7A). To evaluate the role of HOTAIR in distinguishing between the responding and non-responding patients, ROC curve analysis was performed, and these patients were stratified into a low-HOTAIR-expression group ( $n = 56$ ) and a high-HOTAIR-expression group ( $n = 96$ ), with an established cutoff value (4.01) (Figure S7). The

that high HOTAIR expression was associated with poor overall survival (OS) and recurrence-free survival (RFS) in CRC patients (Figure 7C). Furthermore, we performed Cox regression univariate/multivariate analysis to identify whether HOTAIR or any other clinical parameter was an independent indicator for the OS of CRC patients who received 5FU-based chemotherapy. The results indicated that HOTAIR expression level and distant metastasis status maintained their significance as independent prognostic factors for OS of CRC patients receiving 5FU treatment (Table S1).

#### DISCUSSION

In clinical situations, acquired drug resistance and enhanced metastasis frequently follow chemotherapeutic regimens, leading to treatment failure in tumor patients. Despite the extensive research on chemoresistance, the detailed mechanism underlying this phenomenon remains unclear. lncRNA HOTAIR has been documented as playing a role in the epigenetic regulation of gene transcription in breast cancer<sup>9</sup> and, subsequently, in CRC.<sup>27</sup> In our present study, we found that HOTAIR knockdown suppressed cell viability and



(legend on next page)



reversed 5FU resistance, while overexpression of HOTAIR promoted cell viability and 5FU resistance in CRC. Moreover, the pro-resistance function of HOTAIR was achieved through the epigenetic silencing of miR-218 and the upregulation of VOPPI expression, and subsequently, the activation of the NF- $\kappa$ B/TS pathway (Figure 7D). Most importantly, we demonstrated that high HOTAIR expression was positively associated with poor chemotherapy response in CRC patients receiving 5FU-based chemotherapy.

HOTAIR is an lncRNA frequently overexpressed in solid tumors and associated with cancer cell growth and migration.<sup>28,29</sup> It is reported that HOTAIR could promote migration and epithelial-mesenchymal transition by inhibiting miR-331-3p and miR-7.<sup>30,31</sup> On the other hand, HOTAIR can inhibit  $\kappa$ B $\alpha$  (an inhibitor of NF- $\kappa$ B), which, in turn, induced HOTAIR expression through the SETDB1/STAT3 signaling pathway.<sup>31</sup> In addition, emerging evidence has demonstrated that HOTAIR is a chemotherapeutic target and promotes chemoresistance in various malignancies, including gastric cancer,<sup>32</sup> bladder cancer,<sup>33</sup> and glioblastoma.<sup>34</sup> However, the specific role of this important lncRNA in the drug resistance of CRC is not fully understood. In this study, we determined that HOTAIR expression was significantly increased in the HT29 5FU-R cells compared with the parental cells. Moreover, after knockdown of HOTAIR, the 5FU resistance was sufficiently reversed with the significantly decreased proliferation and invasion ability, and increased apoptosis rate; overexpression of HOTAIR showed opposite regulative effects. These results indicated that HOTAIR might contribute to the 5FU-induced chemoresistance in CRC cells.

Previous research has found that HOTAIR negatively regulated the expression of miRNAs mainly by functioning as a competing endogenous RNA (ceRNA) sponge, such as miR-145 and miR-331-3p in breast and gastric cancer, respectively.<sup>35</sup> In contrast to these studies, our work identified that HOTAIR could epigenetically suppress miR-218 expression through EZH2 in CRC cells. Downregulation of miR-218 is a consequence of the epigenetic silencing of its host genes; however, the underlying mechanism is unclear. Li et al.,<sup>19</sup> Fu et al.,<sup>36</sup> and Guan et al.<sup>24</sup> found that miR-218 was downregulated due to the epigenetic silencing of miR-218-2 and its host gene, SLIT3, in pancreatic, liver, and thyroid cancer patients, while Yu et al.<sup>37</sup> and Yang et al.<sup>38</sup> demonstrated that the downregulation of miR-218 is induced by the silencing of miR-218-1 and SLIT2 in prostate cancer and esophageal cancer. Here, we demonstrated for the first time that HOTAIR acted as a bridge to recruit EZH2, which subsequently suppressed miR-

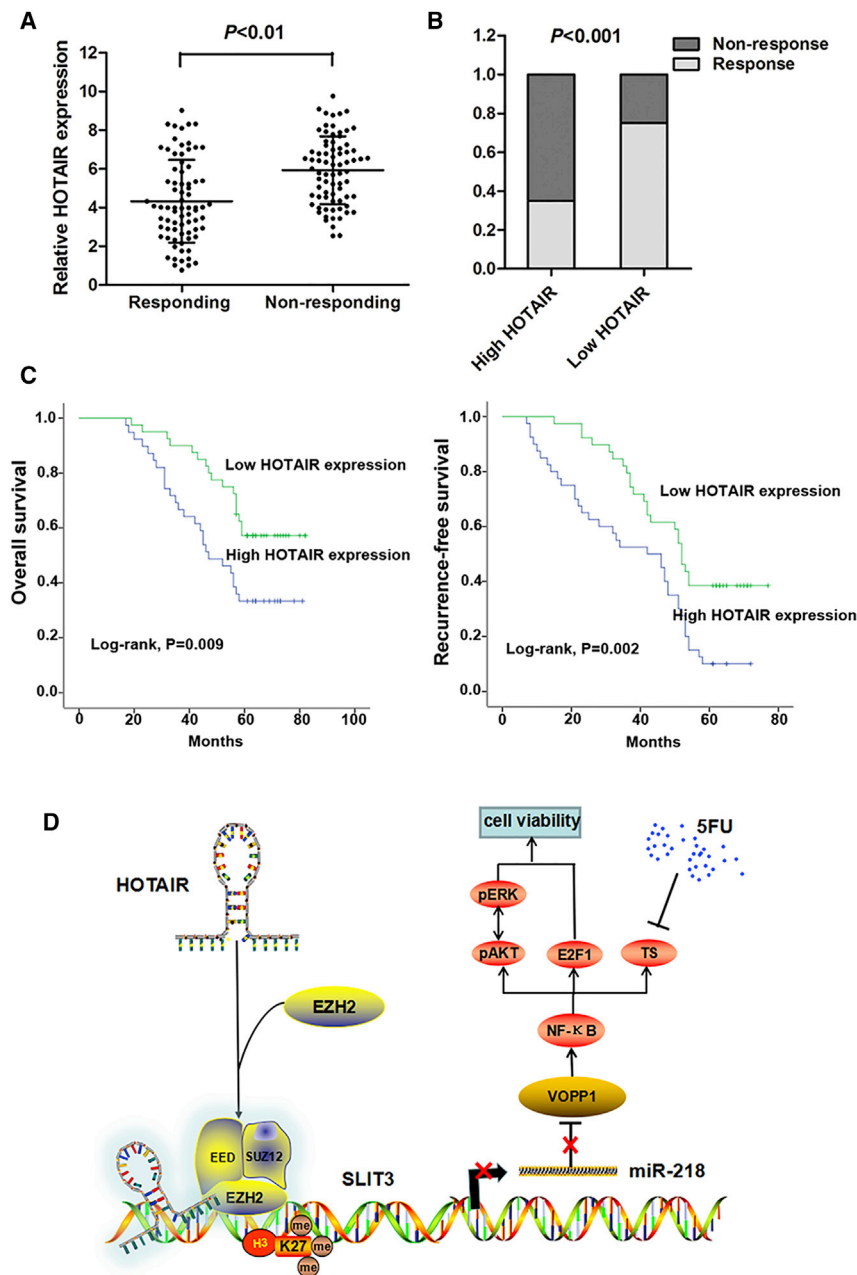
218-2 expression through directly targeting its promoter in CRC. This regulatory pattern is dramatically different from the traditional role of lncRNA as an miRNA sponge. Thus, our present study determined a novel upstream regulatory pattern of miR-218, which might explain how HOTAIR confers a chemoresistant function in CRC.

Generally, miRNAs negatively regulate their mRNA target in a sequence-specific binding manner.<sup>13</sup> In this study, VOPPI was identified as a direct functional target of miR-218 in CRC, which has been previously reported in gastric cancer<sup>39</sup> and glioma.<sup>26</sup> VOPPI was reported to be involved in the regulation of NF- $\kappa$ B transcription activity and contribute to apoptosis resistance. Activation of NF- $\kappa$ B translocation increased cell-cycle progression and downregulated the transcriptional factor E2F-1,<sup>40</sup> which activated the transcription of multiple enzymes, including TS, the critical target for 5FU treatment.<sup>41</sup> This was further reaffirmed in our study. A previous report demonstrated a close positive correlation between TS and E2F-1 expression in primary CRC tissues.<sup>42</sup> Based on these observations, we hypothesized that HOTAIR and miR-218 can inhibit NF- $\kappa$ B translocation by inducing cell viability and downregulating TS in CRC cells. Consistent with our expectations, the expression of NF- $\kappa$ B-p65, E2F-1, pERK, pAKT, and TS were significantly decreased after knockdown of HOTAIR, while they were upregulated by transfection of pHOTAIR. Moreover, the NF- $\kappa$ B signaling was inactivated by miR-218 overexpression and activated by inhibition of miR-218. Collectively, our results revealed that HOTAIR enhanced CRC carcinogenesis through the suppression of miR-218 expression and the activation of NF- $\kappa$ B signaling.

5FU exerts antitumor activity by inducing a state of thymidylate deficiency and creating imbalances in the nucleotide pool, which leads to impaired DNA replication, transcription, and repair and subsequent cell death.<sup>43</sup> However, 5FU resistance has been frequently observed. It is reported that a large proportion of an injected dose of 5FU is degraded by the extracellular enzyme dihydropyrimidine dehydrogenase, inducing 5FU resistance.<sup>44</sup> Moreover, 5FU is a prodrug that requires several intracellular enzymatic steps before it is converted to its other active metabolites, such as fluorodeoxyuridine (FUDR) and fluorodeoxyuridine monophosphate (FUDR-MP). FUDR-MP is the primary active metabolite that inhibits the enzyme TS, a key enzyme in the creation of the DNA nucleotide thymidine.<sup>45</sup> Inhibition of TS prevents conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which, in turn, impairs DNA synthesis in the S phase of the cellular replication cycle.<sup>3</sup> In addition, thymidine

#### Figure 6. HOTAIR Contributes to 5FU Resistance through the Suppression of miR-218 and the Activation of NF- $\kappa$ B/TS Signaling

(A) qRT-PCR showed that HOTAIR was upregulated, while miR-218 was downregulated in HT29 5FU-R cells, compared to parental cells. (B and C) si-HOTAIR (B) or miR-218 mimics (C) promoted 5FU-induced cell cytotoxicity in HT29 5FU-R cells. (D) The enhanced susceptibility to 5FU induced by siHOTAIR-1 was abrogated by anti-miR-218 in HT29 5FU-R cells. (E) VOPPI was upregulated, and the NF- $\kappa$ B pathway was activated in HT29 5FU-R cells, compared with parental cells. (F) NF- $\kappa$ B signaling was dramatically inactivated by transfection with siHOTAIR-1 or miR-218 in HT29 5FU-R cells. (G) VOPPI knockdown also inactivated NF- $\kappa$ B signaling in HT29 5FU-R cells. (H) Ectopic expression of VOPPI reversed the miR-218-induced enhanced susceptibility to 5FU in HT29 5FU-R subline. (I and J) HOTAIR transfection was followed by less decreased viable cells, compared to the negative vector; however, co-transfection with miR-218 mimics partially reversed this effect in HT29 (I) and SW480 cells (J). (K) Immunofluorescence analysis showed that 5FU treatment suppressed the expression of TS protein in HT29 cells. However, this suppression was significantly relieved when the cells were transfected with pHOTAIR or anti-miR-218. Error bars represent SD. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 7. A High HOTAIR Expression Level Is Associated with Poor Response to First-Line 5FU Treatment in CRC Patients**

(A) qRT-PCR showed that HOTAIR expression was significantly increased in CRC patients showing response to 5FU treatment, compared to patients showing no response. (B) The proportion of patients showing response to 5FU treatment is significantly lower in the high-HOTAIR-expressing group than in the low-HOTAIR-expressing group. (C) Kaplan-Meier survival analysis showed that patients with high HOTAIR expression were associated with poor OS and RFS. (D) Proposed model of HOTAIR function in regulating CRC cell viability and as a promoter to 5FU resistance.

leading to MSI occur in 10%–20% of CRCs.<sup>49</sup> In addition, interference of pathways involved in 5FU-induced apoptosis may also have resulted in resistance. Currently, the correlation between lncRNA HOTAIR expression and those enzymes involved in 5FU resistance is yet to be known. In this study, we revealed that HOTAIR promoted TS enzyme expression and restrained 5FU-induced cell cytotoxicity in CRC cells, while this effect was reversed when cells were co-transfected with miR-218 mimics. The  $IC_{50}$  value for 5FU was dramatically upregulated with HOTAIR over-expression. These data strongly support the notion that HOTAIR promoted 5FU resistance through the suppression of miR-218 and the activation of the NF- $\kappa$ B/TS pathway.

Finally, we explored the clinical prognostic and chemotherapeutic significance of HOTAIR in patients who received standard 5FU-based treatment. HOTAIR was reported to act as a prognostic circulating marker and potential therapeutic target in patients with tumor diseases,<sup>50</sup> while its chemotherapeutic value has rarely been discussed in clinical research. In this study, we found that high HOTAIR expression was negatively associated with 5FU-based chemotherapy response and positively associated with shorter OS and RFS in CRC patients receiving 5FU-based

phosphorylase (TP) is an intracellular enzyme that can reversibly convert 5FU to FUDR,<sup>46</sup> while thymidine kinase (TK) converts FUDR to FUDR-MP, and orotate phosphoribosyl transferase (OPRT) may also be required for the activation of 5FU to FUDR-MP.<sup>47</sup> It has been shown that the expression and activity of TP, TK, and OPRT are critical regulators for 5FU resistance and correlate with the sensitivity of CRC cells to the cytotoxic effects of 5FU.<sup>48</sup> Another inherent mechanism of resistance to 5FU is microsatellite instability (MSI) or replication error (RER+). It is reported that sporadic mutations in mismatch repair (MMR) genes (MLH1, MSH2, PMS1, and PMS6)

therapy. This is of considerable therapeutic significance due to the discovery of a HOTAIR inhibition method that may provide a new treatment strategy for improving the chemosensitivity. In the future, a study with a large cohort of samples based on a multi-center, randomized controlled trial is needed to further identify the potential clinical applications of HOTAIR in CRC patients.

In conclusion, our integrated approach demonstrated, for the first time, that HOTAIR contributes to CRC tumorigenesis and 5FU resistance through downregulation of miR-218 and activation of NF- $\kappa$ B

**Table 1. Association of HOTAIR Level and Clinicopathologic Factors in CRC Patients Receiving 5FU-Based Treatment**

|                               | Total n <sup>a</sup> | n <sup>a</sup> (%) for |                       | p Value            |
|-------------------------------|----------------------|------------------------|-----------------------|--------------------|
|                               |                      | High HOTAIR expression | Low HOTAIR expression |                    |
| <b>Gender</b>                 |                      |                        |                       |                    |
| Male                          | 105                  | 64 (42.1)              | 41 (27.0)             | 0.469              |
| Female                        | 47                   | 32 (21.1)              | 15 (9.8)              |                    |
| <b>Age and Year</b>           |                      |                        |                       |                    |
| Median                        |                      | 62                     | 59                    | 0.893              |
| Range                         |                      | 34–85                  | 26–79                 |                    |
| <b>Tumor Differentiation</b>  |                      |                        |                       |                    |
| Well                          | 30                   | 13 (8.6)               | 17 (11.2)             | 0.006 <sup>b</sup> |
| Moderate                      | 71                   | 43 (28.3)              | 28 (18.4)             |                    |
| Poor                          | 51                   | 40 (26.3)              | 11 (7.2)              |                    |
| <b>Dukes' Stages</b>          |                      |                        |                       |                    |
| I and II                      | 67                   | 38 (23.0)              | 29 (19.1)             | 0.144              |
| III and IV                    | 85                   | 58 (38.1)              | 27 (17.8)             |                    |
| <b>Lymph Node Metastasis</b>  |                      |                        |                       |                    |
| Absent                        | 76                   | 45 (29.6)              | 31 (20.4)             | 0.313              |
| Present                       | 76                   | 51 (33.6)              | 25 (16.4)             |                    |
| <b>Distant Metastasis</b>     |                      |                        |                       |                    |
| Absent                        | 116                  | 67 (44.1)              | 49 (32.2)             | 0.013 <sup>b</sup> |
| Present                       | 36                   | 29 (19.1)              | 7 (4.6)               |                    |
| <b>5FU-Based Chemotherapy</b> |                      |                        |                       |                    |
| FOLFOX <sup>c</sup>           | 87                   | 55 (36.2)              | 32 (21.0)             | 0.203              |
| FOLFIRI <sup>d</sup>          | 44                   | 31 (20.4)              | 13 (8.6)              |                    |
| 5FU/LV <sup>e</sup>           | 21                   | 10 (6.6)               | 11 (7.2)              |                    |
| <b>Tumor Size</b>             |                      |                        |                       |                    |
| Small (<3 cm)                 | 106                  | 59 (38.8)              | 47 (31.0)             | 0.004 <sup>b</sup> |
| Large (≥3 cm)                 | 46                   | 37 (24.3)              | 9 (5.9)               |                    |

<sup>a</sup>Number.<sup>b</sup>Statistically significant difference.<sup>c</sup>FOLFOX, 5FU + oxaliplatin + leucovorin.<sup>d</sup>FOLFIRI, 5FU + leucovorin + irinotecan.<sup>e</sup>5FU/Lv, 5FU + leucovorin.

signaling. This lncRNA directly recruits EZH2 and suppresses miR-218 by binding to its promoter, which provides a mechanistic foundation for the aberrant VOPPI activation in CRC. This pro-resistant role of HOTAIR was further validated in an independent set of CRC patients who received standard 5FU treatment. Thus, HOTAIR may be a novel prognostic biomarker and therapeutic target in CRC patients. Suppression of HOTAIR could be a future direction for enhancing chemosensitivity to 5FU-based chemotherapy regimens.

## MATERIALS AND METHODS

### Clinical Samples

For clinical parameter analysis, 48 CRC tissues and paired non-cancerous tissues were collected at the Qilu Hospital of Shandong

University between 2009 and 2011. For chemo-response study, 152 primary tissues were collected from the patients who received standard 5FU-based chemotherapy at the Qilu Hospital of Shandong University between 2009 and 2011. All the patients were pathologically confirmed, and the clinical samples were collected before chemotherapy was started. Tumor response was confirmed through computed tomography and evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Among those 152 patients receiving 5FU-based treatment, 76 showed response (CR and PR), and 76 showed no response (SD and PD). OS was defined as the duration from inclusion to death of any cause, and RFS was defined as the duration from inclusion to recurrence of disease or death of any cause. All patients received a standard follow-up with computed tomography of the abdomen after surgery. Written informed consent was obtained from all patients according to the guidelines approved by the Ethics Committee of the Qilu Hospital of Shandong University.

### Cell Culture

The human CRC cell lines HT29, SW480, and FHC cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences in 2014. CRC cell lines were maintained in RPMI 1640 (Thermo Fisher Scientific) containing 10% FBS (fetal bovine serum) (Sigma-Aldrich), 100 U/mL penicillin, and 100 g/mL streptomycin (Life Technologies) at 37°C in 5% CO<sub>2</sub> and 95% air. Normal colon FHC cells were grown in DMEM/F12 medium (DMEM/Nutrient Mixture F-12) with 10% FBS, 10 ng/mL cholera toxin, 5 µg/mL transferrin, 5 µg/mL insulin, 100 ng/mL hydrocortisone, and an additional 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 37°C in 5% CO<sub>2</sub> and 95% air. The cell lines passed the DNA profiling test (short tandem repeat [STR]).

### Development of 5FU-R CRC Cell Lines

The HT29 5FU-R CRC cells were established as described in the [Supplemental Materials and Methods](#).

### RNA Oligoribonucleotides and Cell Transfection

The siRNAs that specifically target human HOTAIR, EZH2, and VOPPI (vesicular, overexpressed in cancer, pro-survival protein 1) mRNAs were designated as siHOTAIR, siEZH2, and siVOPPI, respectively. The coding sequence of VOPPI was amplified, cloned into pCDNA3.1 vector, and then named pCDNA-VOPPI. The lentivirus vector containing HOTAIR short-hairpin RNA (LV-ShHOTAIR) was amplified and cloned by GeneChem. The full-length HOTAIR overexpression plasmid (pHOTAIR) was also purchased from GeneChem. The NC for both miRNA mimics and siRNA, as well as the single-standard negative-control RNA for miRNA inhibitors (anti-NC), was not homologous to any human genome sequences. All RNA oligoribonucleotides were purchased from Ribo-Bio. The sequences are listed in [Table S2](#). The transfection of RNA oligoribonucleotides and plasmid was performed using Lipofectamine 2000 (Invitrogen), as previously described.<sup>20</sup>

### RNA Extraction, Reverse Transcription, and qRT-PCR

Total RNA was isolated from primary tumors or CRC cell lines using TRIzol reagent (Invitrogen). For miR-218 quantification, the cDNA was synthesized from 200 ng extracted total RNA using the PrimeScript RT Reagent Kit (TaKaRa Bio) and amplified by qRT-PCR with a SYBR Premix Ex Taq Kit (TaKaRa Bio). U6 was used for normalization. For lncRNA and mRNA detection, RNA was reverse-transcribed with a random primer using a TaKaRa Reverse Transcription Kit and amplified with the SYBR Green Kit (TaKaRa Bio) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as internal control. The  $2^{-\Delta\Delta C_t}$  method was used to determine the relative quantification of gene expression levels. The primer sequences are shown in Table S2.

### Cell Viability, Cell-Cycle, and Colony Formation Assays

These assays were performed as described in the [Supplemental Materials and Methods](#).

### Dual-Luciferase Reporter Assays

The luciferase reporter assay was performed as described in the [Supplemental Materials and Methods](#).

### RIP and ChIP

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). The RNAs were immunoprecipitated using anti-EZH2 (Cell Signaling Technology) antibody. Final analysis was performed using qRT-PCR and shown as fold enrichment of HOTAIR. The RIP RNA fraction Ct value was normalized to the input RNA fraction Ct value. ChIP was performed using the EZ ChIP Chromatin Immunoprecipitation Kit (Millipore), according to the manufacturer's protocol. Briefly, cross-linked chromatin was sonicated into 200-1,000-bp fragments. The chromatin was immunoprecipitated by using anti-EZH2 (Cell Signaling Technology) and anti-H3K27me3 (Millipore) antibodies. qRT-PCR was conducted to detect the relative enrichment of the miR-218-2 promoter. Primers are listed in Table S2.

### Immunofluorescence Analysis

HT29 cells were grown to 40% to 50% confluence and then transfected with 100 nM of si-HOTAIR or anti-miR-218. After 48 hr of incubation, the cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) for 20 min. The cells were then blocked with 10% goat serum in PBS for 1 hr. Cells were incubated with primary anti-Ki-67 (Santa Cruz Biotechnology) or anti-thymidylate synthase (TS; Abcam) overnight at 4°C and then incubated with the appropriate rhodamine-conjugated secondary antibody for 1 hr. The cells were then washed and incubated with DAPI (Invitrogen) for nuclear staining. The slides were visualized for immunofluorescence with a laser-scanning Olympus microscope.

### Antibodies and Western Blot

A total of 25 µg protein from each sample was separated on 10% Bis-Tris polyacrylamide gel through electrophoresis and then

blotted onto PVDF (polyvinylidene fluoride) membranes (Millipore). Blots were immunostained with primary antibodies, including rabbit anti-EZH2 (Cell Signaling Technology), anti-VOPP1 (Santa Cruz), anti-Ki-67 (Santa Cruz), anti-p65-NF-κB (Cell Signaling Technology), anti-E2F-1 (Cell Signaling Technology), anti-pAKT (Phospho-Thr308; Cell Signaling Technology), anti-pERK (Phospho-Thr980; Cell Signaling Technology), anti-TS (Abcam), and anti-β-actin (Cell Signaling Technology) at 4°C overnight and with secondary antibody at room temperature for 1 hr. Immunoblots were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). β-actin was used as the internal control.

### Statistical Analysis

For CRC versus normal cell lines and CRC tissue versus adjacent non-tumor tissue, differences in mean expression were determined using Student's t test. The survival curves of CRC patients were estimated via the Kaplan-Meier method, and the difference in survival curves was estimated using log-rank testing. Receiver operating characteristic (ROC) curves were established to evaluate the potential value of HOTAIR in discriminating responding patients from non-responding patients. The correlation analysis was evaluated using the Spearman test. The results were considered statistically significant at  $p < 0.05$ . Error bars in figures represent SD. Statistical analyses were performed with GraphPad Prism (v5.01) software.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, seven figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.omtn.2017.07.007>.

### AUTHOR CONTRIBUTIONS

C.W., P.L., and X.Z. acquired the data and created a draft of the manuscript; P.L. and T.L. collected clinical samples and performed the in vitro assays; L.W. and L.D. analyzed and interpreted data and statistical analysis; Y.Y. and C.L. provided technical and material support; C.W. and P.L. approved the final version of the manuscript.

### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

### ACKNOWLEDGMENTS

C.W. received support from the National Natural Science Foundation of China (81472025) and the Taishan Scholar Foundation; X.Z. received support from the National Natural Science Foundation of China (81301506), the Research Fund for the Doctoral Program of Higher Education of China (20130131120067), and the Shandong Key Research and Development Program (2015GSF118075); L.W. received support from the Outstanding Young Scientist Research Award Fund of Shandong Province (BS2014yy002) and the Shandong Key Research and Development Program (2015GSF118167); Y.Y. received support from the Fundamental Research Funds of Shandong University (2014QLKY03).

## REFERENCES

- Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. *CA Cancer J. Clin.* 65, 87–108.
- Goldberg, R.M., Sargent, D.J., Morton, R.F., Fuchs, C.S., Ramanathan, R.K., Williamson, S.K., Findlay, B.P., Pitot, H.C., and Alberts, S.R. (2004). A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J. Clin. Oncol.* 22, 23–30.
- Chu, E., Callender, M.A., Farrell, M.P., and Schmitz, J.C. (2003). Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. *Cancer Chemother. Pharmacol.* 52 (Suppl. 1), S80–S89.
- Alberts, S.R., Horvath, W.L., Sternfeld, W.C., Goldberg, R.M., Mahoney, M.R., Dakhil, S.R., Levitt, R., Rowland, K., Nair, S., Sargent, D.J., and Donohue, J.H. (2005). Oxaliplatin, fluorouracil, and leucovorin for patients with unresectable liver-only metastases from colorectal cancer: a North Central Cancer Treatment Group phase II study. *J. Clin. Oncol.* 23, 9243–9249.
- Douillard, J.Y., Cunningham, D., Roth, A.D., Navarro, M., James, R.D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., et al. (2000). Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 355, 1041–1047.
- Alhopuro, P., Alazouzi, H., Sammalkorpi, H., Dávalos, V., Salovaara, R., Hemminki, A., Järvinen, H., Mecklin, J.P., Schwartz, S., Jr., Aaltonen, L.A., and Arango, D. (2005). SMAD4 levels and response to 5-fluorouracil in colorectal cancer. *Clin. Cancer Res.* 11, 6311–6316.
- Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. *Nat. Rev. Genet.* 10, 155–159.
- The ENCODE Project Consortium (2007). Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447, 799–816.
- Gupta, R.A., Shah, N., Wang, K.C., Kim, J., Horlings, H.M., Wong, D.J., Tsai, M.C., Hung, T., Argani, P., Rinn, J.L., et al. (2010). Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464, 1071–1076.
- Zhang, Y., Zhang, K., Luo, Z., Liu, L., Wu, L., and Liu, J. (2016). Circulating long non-coding HOX transcript antisense intergenic ribonucleic acid in plasma as a potential biomarker for diagnosis of breast cancer. *Thorac. Cancer* 7, 627–632.
- Li, C.Y., Liang, G.Y., Yao, W.Z., Sui, J., Shen, X., Zhang, Y.Q., Peng, H., Hong, W.W., Ye, Y.C., Zhang, Z.Y., et al. (2016). Integrated analysis of long non-coding RNA competing interactions reveals the potential role in progression of human gastric cancer. *Int. J. Oncol.* 48, 1965–1976.
- Svoboda, M., Slyskova, J., Schneiderova, M., Makovicky, P., Bielik, L., Levy, M., Lipska, L., Hemmelova, B., Kala, Z., Protivankova, M., et al. (2014). HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients. *Carcinogenesis* 35, 1510–1515.
- Bayoumi, A.S., Sayed, A., Broskova, Z., Teoh, J.P., Wilson, J., Su, H., Tang, Y.L., and Kim, I.M. (2016). Crosstalk between long noncoding RNAs and microRNAs in health and disease. *Int. J. Mol. Sci.* 17, 356.
- Tatarano, S., Chiyomaru, T., Kawakami, K., Enokida, H., Yoshino, H., Hidaka, H., Yamasaki, T., Kawahara, K., Nishiyama, K., Seki, N., and Nakagawa, M. (2011). miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. *Int. J. Oncol.* 39, 13–21.
- Tie, J., Pan, Y., Zhao, L., Wu, K., Liu, J., Sun, S., Guo, X., Wang, B., Gang, Y., Zhang, Y., et al. (2010). miR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. *PLoS Genet.* 6, e1000879.
- Wu, D.W., Cheng, Y.W., Wang, J., Chen, C.Y., and Lee, H. (2010). Paxillin predicts survival and relapse in non-small cell lung cancer by microRNA-218 targeting. *Cancer Res.* 70, 10392–10401.
- He, X., Dong, Y., Wu, C.W., Zhao, Z., Ng, S.S., Chan, F.K., Sung, J.J., and Yu, J. (2013). MicroRNA-218 inhibits cell cycle progression and promotes apoptosis in colon cancer by downregulating BMI1 polycomb ring finger oncogene. *Mol. Med.* 18, 1491–1498.
- Alajez, N.M., Lenarduzzi, M., Ito, E., Hui, A.B., Shi, W., Bruce, J., Yue, S., Huang, S.H., Xu, W., Waldron, J., et al. (2011). miR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res.* 71, 2381–2391.
- Li, C.H., To, K.F., Tong, J.H., Xiao, Z., Xia, T., Lai, P.B., Chow, S.C., Zhu, Y.X., Chan, S.L., Marquez, V.E., and Chen, Y. (2013). Enhancer of zeste homolog 2 silences microRNA-218 in human pancreatic ductal adenocarcinoma cells by inducing formation of heterochromatin. *Gastroenterology* 144, 1086–1097.e9.
- Li, P.L., Zhang, X., Wang, L.L., Du, L.T., Yang, Y.M., Li, J., and Wang, C.X. (2015). MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5. *Carcinogenesis* 36, 1484–1493.
- Li, P., Zhang, X., Wang, H., Wang, L., Liu, T., Du, L., Yang, Y., and Wang, C. (2017). MALAT1 is associated with poor response to oxaliplatin-based chemotherapy in colorectal cancer patients and promotes chemoresistance through EZH2. *Mol. Cancer Ther.* 16, 739–751.
- Ishikawa, S., Hayashi, H., Kinoshita, K., Abe, M., Kuroki, H., Tokunaga, R., Tomiyasu, S., Tanaka, H., Sugita, H., Arita, T., et al. (2014). Statins inhibit tumor progression via an enhancer of zeste homolog 2-mediated epigenetic alteration in colorectal cancer. *Int. J. Cancer* 135, 2528–2536.
- Liu, Y.L., Gao, X., Jiang, Y., Zhang, G., Sun, Z.C., Cui, B.B., and Yang, Y.M. (2015). Expression and clinicopathological significance of EED, SUZ12 and EZH2 mRNA in colorectal cancer. *J. Cancer Res. Clin. Oncol.* 141, 661–669.
- Guan, H., Wei, G., Wu, J., Fang, D., Liao, Z., Xiao, H., Li, M., and Li, Y. (2013). Down-regulation of miR-218-2 and its host gene SLIT3 cooperate to promote invasion and progression of thyroid cancer. *J. Clin. Endocrinol. Metab.* 98, E1334–E1344.
- Park, S., and James, C.D. (2005). ECop (EGFR-coamplified and overexpressed protein), a novel protein, regulates NF-kappaB transcriptional activity and associated apoptotic response in an IkappaBalpha-dependent manner. *Oncogene* 24, 2495–2502.
- Xia, H., Yan, Y., Hu, M., Wang, Y., Wang, Y., Dai, Y., Chen, J., Di, G., Chen, X., and Jiang, X. (2013). miR-218 sensitizes glioma cells to apoptosis and inhibits tumorigenicity by regulating ECOP-mediated suppression of NF-kB activity. *Neuro-oncol.* 15, 413–422.
- Kogo, R., Shimamura, T., Mimori, K., Kawahara, K., Imoto, S., Sudo, T., Tanaka, F., Shibata, K., Suzuki, A., Komune, S., et al. (2011). Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers. *Cancer Res.* 71, 6320–6326.
- Kim, K., Jutooru, I., Chadalapaka, G., Johnson, G., Frank, J., Burghardt, R., Kim, S., and Safe, S. (2013). HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. *Oncogene* 32, 1616–1625.
- Zhou, X., Chen, J., and Tang, W. (2014). The molecular mechanism of HOTAIR in tumorigenesis, metastasis, and drug resistance. *Acta Biochim. Biophys. Sin. (Shanghai)* 46, 1011–1015.
- Liu, X.H., Sun, M., Nie, F.Q., Ge, Y.B., Zhang, E.B., Yin, D.D., Kong, R., Xia, R., Lu, K.H., Li, J.H., et al. (2014). Lnc RNA HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer. *Mol. Cancer* 13, 92.
- Zhang, H., Cai, K., Wang, J., Wang, X., Cheng, K., Shi, F., Jiang, L., Zhang, Y., and Dou, J. (2014). MiR-7, inhibited indirectly by lincRNA HOTAIR, directly inhibits SETDB1 and reverses the EMT of breast cancer stem cells by downregulating the STAT3 pathway. *Stem Cells* 32, 2858–2868.
- Shang, C., Guo, Y., Zhang, H., and Xue, Y.X. (2016). Long noncoding RNA HOTAIR is a prognostic biomarker and inhibits chemosensitivity to doxorubicin in bladder transitional cell carcinoma. *Cancer Chemother. Pharmacol.* 77, 507–513.
- Zhao, W., Dong, S., Duan, B., Chen, P., Shi, L., Gao, H., and Qi, H. (2015). HOTAIR is a predictive and prognostic biomarker for patients with advanced gastric adenocarcinoma receiving fluorouracil and platinum combination chemotherapy. *Am. J. Transl. Res.* 7, 1295–1302.
- Zhou, X., Ren, Y., Zhang, J., Zhang, C., Zhang, K., Han, L., Kong, L., Wei, J., Chen, L., Yang, J., et al. (2015). HOTAIR is a therapeutic target in glioblastoma. *Oncotarget* 6, 8353–8365.
- Li, J.T., Wang, L.F., Zhao, Y.L., Yang, T., Li, W., Zhao, J., Yu, F., Wang, L., Meng, Y.L., Liu, N.N., et al. (2014). Nuclear factor of activated T cells 5 maintained by HotaIR suppression of miR-568 upregulates S100 calcium binding protein A4 to promote breast cancer metastasis. *Breast Cancer Res.* 16, 454.

36. Fu, W.M., Zhu, X., Wang, W.M., Lu, Y.F., Hu, B.G., Wang, H., Liang, W.C., Wang, S.S., Ko, C.H., Waye, M.M., et al. (2015). Hotair mediates hepatocarcinogenesis through suppressing miRNA-218 expression and activating P14 and P16 signaling. *J. Hepatol.* *63*, 886–895.
37. Yu, J., Cao, Q., Yu, J., Wu, L., Dallol, A., Li, J., Chen, G., Grasso, C., Cao, X., Lonigro, R.J., et al. (2010). The neuronal repellent SLIT2 is a target for repression by EZH2 in prostate cancer. *Oncogene* *29*, 5370–5380.
38. Yang, M., Liu, R., Li, X., Liao, J., Pu, Y., Pan, E., Wang, Y., and Yin, L. (2015). Epigenetic repression of miR-218 promotes esophageal carcinogenesis by targeting ROBO1. *Int. J. Mol. Sci.* *16*, 27781–27795.
39. Gao, C., Zhang, Z., Liu, W., Xiao, S., Gu, W., and Lu, H. (2010). Reduced microRNA-218 expression is associated with high nuclear factor kappa B activation in gastric cancer. *Cancer* *116*, 41–49.
40. Nagaraju, G.P., Alese, O.B., Landry, J., Diaz, R., and El-Rayes, B.F. (2014). HSP90 inhibition downregulates thymidylate synthase and sensitizes colorectal cancer cell lines to the effect of 5FU-based chemotherapy. *Oncotarget* *5*, 9980–9991.
41. Wilson, P.M., Danenberg, P.V., Johnston, P.G., Lenz, H.J., and Ladner, R.D. (2014). Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. *Nat. Rev. Clin. Oncol.* *11*, 282–298.
42. Banerjee, D., Gorlick, R., Liefshitz, A., Danenberg, K., Danenberg, P.C., Danenberg, P.V., Klimstra, D., Jhanwar, S., Cordon-Cardo, C., Fong, Y., et al. (2000). Levels of E2F-1 expression are higher in lung metastasis of colon cancer as compared with hepatic metastasis and correlate with levels of thymidylate synthase. *Cancer Res.* *60*, 2365–2367.
43. Hammond, W.A., Swaika, A., and Mody, K. (2016). Pharmacologic resistance in colorectal cancer: a review. *Ther. Adv. Med. Oncol.* *8*, 57–84.
44. Longley, D.B., Harkin, D.P., and Johnston, P.G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer* *3*, 330–338.
45. Danenberg, P.V. (1977). Thymidylate synthetase - a target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* *473*, 73–92.
46. Panczyk, M. (2014). Pharmacogenetics research on chemotherapy resistance in colorectal cancer over the last 20 years. *World J. Gastroenterol.* *20*, 9775–9827.
47. Koopman, M., Venderbosch, S., van Tinteren, H., Ligtenberg, M.J., Nagtegaal, I., Van Krieken, J.H., and Punt, C.J. (2009). Predictive and prognostic markers for the outcome of chemotherapy in advanced colorectal cancer, a retrospective analysis of the phase III randomised CAIRO study. *Eur. J. Cancer* *45*, 1999–2006.
48. Houghton, J.A., and Houghton, P.J. (1983). Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. *Eur. J. Cancer Clin. Oncol.* *19*, 807–815.
49. Carethers, J.M., Smith, E.J., Behling, C.A., Nguyen, L., Tajima, A., Doctolero, R.T., Cabrera, B.L., Goel, A., Arnold, C.A., Miyai, K., and Boland, C.R. (2004). Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. *Gastroenterology* *126*, 394–401.
50. Botti, G., Marra, L., Malzone, M.G., Anniciello, A., Botti, C., Franco, R., and Cantile, M. (2017). LncRNA HOTAIR as prognostic circulating marker and potential therapeutic target in patients with tumor diseases. *Curr. Drug Targets* *18*, 27–34.