Molecular Cancer Therapeutics

MALAT1 Is Associated with Poor Response to Oxaliplatin-Based Chemotherapy in Colorectal Cancer Patients and Promotes Chemoresistance through EZH2

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Abstract

A major reason for oxaliplatin chemoresistance in colorectal cancer is the acquisition of epithelial–mesenchymal transition (EMT) in cancer cells. The long noncoding RNA (lncRNA), MALAT1, is a highly conserved nuclear ncRNA and a key regulator of metastasis development in several cancers. However, its role in oxaliplatin-induced metastasis and chemoresistance is not well known. In this study, we aim to investigate the prognostic and therapeutic role of lncRNA MALAT1 in colorectal cancer patients receiving oxaliplatin-based therapy and further explore the potential transcriptional regulation through interaction with EZH2 based on the established HT29 oxaliplatin-resistant cells. Our results showed that high MALAT1 expression was associated with reduced patient survival and poor response to oxaliplatin-based chemotherapy in advanced colorectal cancer patients. Oxaliplatin-resistant colorectal cancer cells exhibited high MALAT1 expression and EMT. LncRNA MALAT1 knockdown enhances E-cadherin expression and inhibits oxaliplatin-induced EMT in colorectal cancer cells. EZH2 is highly expressed and associated with the 3′ end region of lncRNA MALAT1 in colorectal cancer, and this association suppressed the expression of E-cadherin. Furthermore, targeted inhibition of MALAT1 or EZH2 reversed EMT and chemoresistance induced by oxaliplatin. Finally, the interaction between lncRNA MALAT1 and miR-218 was observed, which further indicated its prognostic value in patients who received standard FOLFOX (oxaliplatin combine with 5-fluorouracil and leucovorin) treatment. In conclusion, this study illuminates the prognostic role of lncRNA MALAT1 in colorectal cancer patients receiving oxaliplatin-based treatment and further demonstrates how lncRNA MALAT1 confers a chemoresistant function in colorectal cancer. Thus, lncRNA MALAT1 may serve as a promising prognostic and therapeutic target for colorectal cancer patients. *Mol Cancer Ther; 16(4); 739–51. ©2017 AACR.*

Introduction

Colorectal cancer is a leading cause of cancer-related deaths in the world. It is the second- and third-most commonly diagnosed cancer in females and males, respectively, and more than 1.2 million patients are diagnosed with colorectal cancer every year (1, 2). Currently, oxaliplatin-based chemotherapy after surgical resection is one of the most frequently used therapeutic strategies (3). Its use in combination with 5-fluorouracil (5-FU) and leucovorin (FOLFOX) has led to response rates >50% and median survival approaching 2 years for metastatic colorectal cancer (4). However, a large proportion of patients receiving chemotherapy finally become metastatic and chemoresistant, and this has been a key barrier to the efficacy of colorectal cancer treatment (5). A major reason for colorectal cancer chemoresistance is the enhanced invasion and metastasis of cancer cells, such as the cell acquisition of epithelial–mesenchymal transition (EMT; refs. 6, 7). Revealing the underlying mechanism and finding new therapeutic and prognostic targets are necessary for developing effective therapies for colorectal cancer patients.

Long noncoding RNAs (lncRNA) are most commonly defined as the RNA transcripts of more than 200 nucleotides (nt) and located in nuclear or cytosolic fractions with no protein-coding capacity (8). Recent studies have demonstrated that lncRNAs play important roles in carcinogenesis and cancer metastasis, and some lncRNAs functioned as oncogenes, tumor-suppressor genes, or both, depending on the circumstances (9). The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was first demonstrated by Ji and colleagues as an oncogene in non–small cell lung cancer through the promotion of cell metastasis and invasion (10). LncRNA MALAT1 can interact with Pc2 (Polycomb 2), a component of the Polycomb Repressive Complex 1 (PRC1). This interaction controls the re-localization of growth control genes between polycomb bodies and interchromatin granules (11). Subsequent studies reported that lncRNA MALAT1 expression was an independent prognostic parameter and had a role in cell metastasis and EMT processes in bladder cancer, renal cancer, gastric cancer, and colorectal cancer (12–15). Xu and

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colleagues demonstrated that a motif of the 3′ end MALAT1 gene played an important role in the biological processes of human colorectal malignancies (16). Despite the research advances of lncRNA MALAT1, the role of lncRNA MALAT1 in colorectal cancer development and chemoresistance is still poorly understood, and new research to uncover the potential mechanism is urgently needed.

EZH2, a critical component of polycomb repressive complex 2 (PRC2), functions as a histone H3 Lysine 27 (H3K27) methyltransferase in target gene promoters and inhibits specific gene expression (17). EZH2 has frequently been found to be overexpressed in a variety of human cancers (13, 18). In addition, EZH2 silenced E-cadherin during the EMT processes of cancer cells and gave rise to cancer progression (19). More importantly, studies demonstrated that MALAT1 interacted with EZH2 and facilitated its recruitment to gene promoter in renal and gastric cancer (13, 14), but it was not reported in colorectal cancer. Thus, the study on the regulatory mode between lncRNA MALAT1 and EZH2 in colorectal cancer is a meaningful work.

In this work, we focused on the prognostic role of lncRNA MALAT1 in colorectal cancer patients receiving oxaliplatin-based chemotherapy. On this basis, we investigated the underlying function of lncRNA MALAT1 on oxaliplatin-induced EMT and chemoresistance of colorectal cancer cells. Moreover, we revealed the potential mechanism involving the miR-218 interaction and special E-cadherin silencing by EZH2-induced gene methylation.

Materials and Methods

Clinical samples

For clinical parameter analysis, 68 colorectal cancer tissues and paired adjacent noncancerous tissues were collected at Qilu Hospital of Shandong University between 2008 and 2011. For chemoresponse study, 221 serum samples and 48 primary tissues were collected from the patients who received standard oxaliplatin-based chemotherapy at Qilu Hospital of Shandong University between 2008 and 2011. To explore the interaction between MALAT1 and miR-218, an independent set of 46 primary cancer tissue samples from patients who received standard FOLFOX chemotherapy were also collected at Qilu Hospital of Shandong University between 2011 and 2014. All the patients were pathologically confirmed, and the clinical samples were collected before chemotherapy was started. Patients were classified according to the World Health Organization criteria and staged according to the tumor–node–metastasis classification. Tumor response was confirmed through CT and evaluated according to the RECIST criteria as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Overall survival (OS) was defined as the duration from inclusion to death of any cause, and progression-free survival (PFS) was defined as the duration from inclusion to PD or death of any cause. All patients received a standard follow-up with CT of the abdomen after surgery. Written informed consent was obtained from all patients according to the guidelines approved by the Ethics Committee of Qilu Hospital, Shandong University.

Cell culture

The human colorectal cancer cell lines HT29, SW480, SW620, and human fetal normal colonic cell (FHC) were obtained from the Type Culture Collection of the Chinese Academy of Sciences in 2014. All colorectal cancer cell lines were maintained in RPMI 1640 (Thermo Fisher Scientific) containing 10% FBS (Sigma-Aldrich), 100 U/mL penicillin, and 100 g/mL streptomycin (Life Technologies) at 37°C in 5% CO_2 and 95% air. Normal colon FHC cells were grown in DMEM/F12 medium with 10% FBS, 10 ng/mL cholera toxin, 5 μg/mL transferrin, 5 μg/mL insulin, 100 ng/mL hydrocortisone, and an extra 10 mmol/L of 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid at 37°C in 5% CO_2 and 95% air. The cell authenticity was determined by short tandem repeat analysis technology (Cell ID System, Promega).

Development of oxaliplatin-resistant colorectal cancer cell lines

The HT29 oxaliplatin-resistant (OxR) colorectal cancer cells were established as described in Supplementary Methods.

Total RNA and protein extraction

Total RNA and protein were extracted as described in Supplementary Methods.

Quantitative real-time PCR and RT-qPCR directly applied in serum

For colorectal cancer tissues and cell lines, the cDNA was synthesized from 200 ng extracted total RNA using the PrimeScript RT reagent Kit (Takara Bio Company) and amplified by RT-qPCR with an SYBR Green kit (Takara Bio Company) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with the housekeeping gene *GAPDH* as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantification of gene expression levels. All the premier sequences were synthesized by RiboBio, and their sequences are shown in Supplementary Table S1.

For cell-free serum MALAT1 detection, we used our previously established RT-qPCR-D (RT-qPCR directly applied in serum) method without RNA extraction (20). Briefly, we prepared the 2×preparation buffer that contained 2.5% Tween 20 (EMD Chemicals), 50 mmol/L Tris (Sigma-Aldrich), and 1 mmol/L EDTA (Sigma-Aldrich) as Asaga and colleagues described (21). First, 5 μL of serum were mixed with an equal volume of 2×preparation buffer. Subsequently, the above mixture was reverse transcribed (RT) in triplicates in a 20 μL reaction volume. Finally, the RT product was diluted 10-fold and centrifuged at 16,000 *g* for 5 minutes, and a 5-μL supernatant solution was used as the cDNA template for qPCR. The reagents and reaction conditions were the same as those for RT-qPCR.

Knockdown of MALAT1 and EZH2 in colorectal cancer cells

Colorectal cancer cells were plated in a 6-well plate in RIPM 1640 supplemented with 10% FBS and cultured until 50% to 70% confluent. SiRNAs were mixed with Lipofectamine 2000 (Invitrogen) in reduced serum medium (Opti-MEM, Gibco) according to the manufacturer's instructions, and final concentration of siRNAs was 100 nmol/L. Knockdown effect was examined by RT-qPCR using RNA extracted 48 hours after transfection. The siRNA sequences of lncRNA-MALAT1, EZH2, and negative control used in this study are listed in Supplementary Table S1.

Cell migration and invasion assays

Cell migration and invasion were assessed with Boyden chambers or modified Boyden chambers according to the manufacturer's protocol (Becton Dickinson Labware).

Cell viability assay

The cell viability assay was performed as described in Supplementary Methods.

RNA immunoprecipitation

An RNA immunoprecipitation (RIP) experiment was performed to investigate whether ribonucleoprotein complex contained lncRNA MALAT1 and its potential binding protein (EZH2) in colorectal cancer cells. The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used according to the manufacturer's instructions. The RNAs were immunoprecipitated using anti-EZH2 (Cell Signaling Technology) antibody. Total RNA and controls were also assayed to demonstrate that the detected signals were from RNAs specifically binding to EZH2. The final analysis was performed using RT-qPCR and shown as the fold enrichment of MALAT1. The RIP RNA fraction Ct value was normalized to the input RNA fraction Ct value. Primers are listed in Supplementary Table S1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (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 to 1,000 bp fragments. The chromatin was immunoprecipitated using anti-EZH2 (Cell Signaling Technology) and anti-H3K27me3 (Millipore) antibodies. Normal human immunoglobulin G (IgG) was used as a negative control. RT-qPCR was conducted to detect the relative enrichment according to the method described above. Primers are listed in Supplementary Table S1.

Immunofluorescence analysis

HT29 cells were grown to 40% to 50% confluence and then transfected with 100 nmol/L of si-MALAT1 or si-EZH2. After 48 hours of incubation, the cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) for 20 minutes. The cells were then blocked with 10% goat serum in PBS for 1 hour. The cells were incubated with primary anti–E-cadherin overnight at 4°C and then incubated with the appropriate rhodamine-conjugated secondary antibody for 1 hour. 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.

Western blot and antibodies

The primary antibodies used for Western blotting were rabbit anti-human EZH2 antibody (1:1,000; Cell Signaling Technology), rabbit anti-human β-actin antibody (1:1,000; Cell Signaling Technology), rabbit anti-human Vimentin antibody (1:1,000; Santa Cruz Biotechnology), and rabbit anti-human E-cadherin antibody (1:1,000; Santa Cruz Biotechnology). Horseradish peroxidase (HRP)–conjugated anti-rabbit antibodies (1:5,000; Santa Cruz Biotechnology) were used as the secondary antibodies. A total of 25 μg protein from each sample was separated on 10% Bis-Tris polyacrylamide gel through electrophoresis and then blotted onto polyvinylidene fluoride membranes (GE Healthcare). Then, the membrane was blocked with 5% (5 g/100 mL) nonfat dry milk (Bio-Rad) in tri-buffered saline plus Tween (TBS-T) buffer for 2 hours. Blots were immunostained with primary antibody at 4°C overnight and with secondary antibody at room temperature for 1 hour. Immunoblots were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Protein levels were normalized to β-actin.

Statistical analysis

For colorectal cancer versus normal cell lines, colorectal cancer serum versus healthy control, and colorectal cancer tissue versus adjacent nontumor tissue, differences were shown in mean expression and were determined using the Student *t* test. The survival curves of colorectal cancer patients were estimated via the Kaplan–Meier method, and the difference in survival curves was estimated using the log-rank testing. The correlation analysis was evaluated by using the Spearman test. The Mann–Whitney *U* test or Kruskal–Wallis test was used for evaluating the pathologic difference among clinical cohort groups. Count dates were described as frequency and examined using the Fishe's exact test. The results were considered statistically significant at $P < 0.05$. Error bars in figures represent SD (standard deviation). Statistical analyses were performed with GraphPad Prism (version 5.01) software.

Results

MALAT1 is overexpressed in colorectal cancer patients and correlated with tumor metastasis

RT-qPCR was used to detect MALAT1 expression levels in cell lines and clinical tissues, normalized to GAPDH. All seven colorectal cancer cell lines expressed higher MALAT1 than the normal FHC cell line, especially in SW620 cells that originated from metastasis colorectal cancer (Fig. 1A). The expression of MALAT1 was significantly increased in 68 colorectal cancer tissues compared with paired noncancerous tissues (Fig. 1B). Moreover, the colorectal cancer tissues in 58.8% (40 of 68) of cases had at least 2-fold higher expression of MALAT1 than paired noncancerous tissues (Fig. 1C). The samples were also used and divided into two groups using a median MALAT1 value of 0.398. We then investigated the association of MALAT1 expression with clinical parameters, including age, gender, differentiation, pathologic tumor classification (pT), pathologic lymph node status (pN), pathologic metastatic status (pM), and stage. The expression of MALAT1 was significantly higher in pT (pT3+pT4), pN (+), pM (+), and high stage (3+4) patients compared with lower stage (1+2) patients (Supplementary Table S2), which indicated that MALAT1 may play its oncogenic role primarily in colorectal cancer with advancing progression, but not in the initial phase. Moreover, OS was significantly shorter in the high MALAT1 expression group than in the low MALAT1 group (Fig. 1D).

High MALAT1 expression is associated with poor response to first-line oxaliplatin treatment in colorectal cancer patients

We next sought to explore the association between MALAT1 and response to treatment in an independent set of 53 serum samples from patients with metastatic colorectal cancer treated with oxaliplatin by using the RT-qPCR-D method. Patients were divided into responding (CR + PR) and nonresponding (SD + PD) groups according to RECIST criteria. We found that the MALAT1 expression level was much higher in patients who did not respond to treatment than those who experienced response to chemotherapy (Fig. 1E). This association between MALAT1 and treatment response was further validated in another set of 48 primary tissue samples from patients receiving oxaliplatin-based treatment (Fig. 1F).

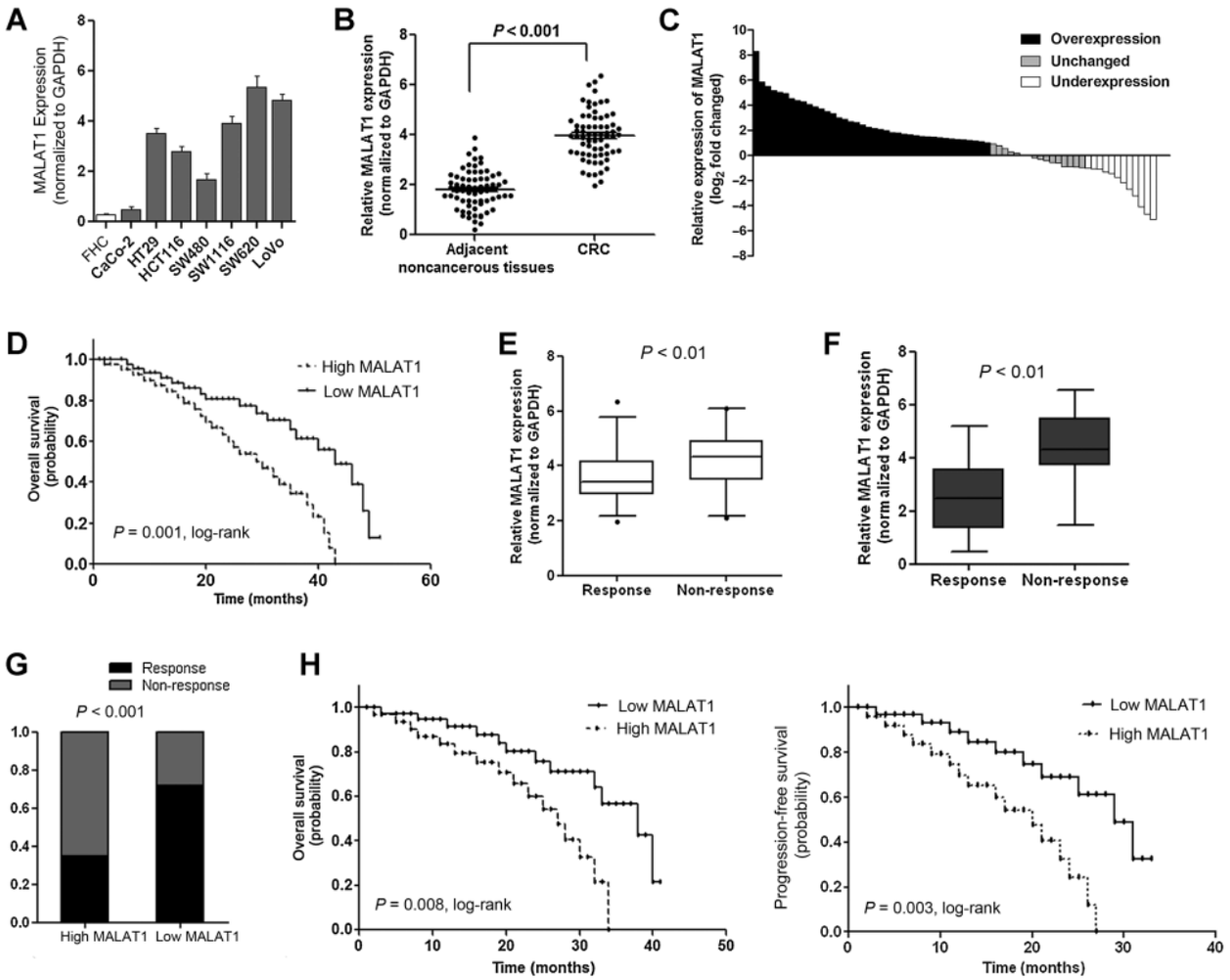

Figure 1.

High MALAT1 expression is correlated with metastasis and associated with poor response to oxaliplatin treatment in colorectal cancer (CRC) patients. **A** and **B,** MALAT1 expression was determined by RT-qPCR and normalized against GAPDH in normal colon FHC cells and 7 colorectal cancer cell lines (**A**) and 68 paired colorectal cancer tissues and adjacent noncancerous tissues (**B**). **C,** The MALAT1 expression level was analyzed using RT-qPCR and expressed as $\log_2$ fold change (colorectal cancer/normal), and the $\log_2$ fold changes were presented as follows: >1, overexpression (40 cases); <1, underexpression (12 cases); the remainder were defined as unchanged (16 cases). **D,** Colorectal cancer primary tissues were divided into a high and a low MALAT1 expression group by using a median value of 0.398, and the association of MALAT1 expression with OS was analyzed with a Kaplan–Meier plot. **E** and **F,** The expression of MALAT1 in responding and nonresponding groups was determined in the primary tissues (**E**) and serum (**F**) of colorectal cancer patients (shown in 10%–90%). **G,** Fifty-three serum samples from colorectal cancer patients were divided into a low and high MALAT1 expression group by establishing an ROC curve. Then, the proportion of patients that responded or did not respond to chemotherapy in the high and low MALAT1 expression group was analyzed ($P < 0.001$, Fisher exact test). **H,** Kaplan–Meier curves for OS (left) and PFS (right) were drawn according to MALAT1 expression and were analyzed by using the log-rank test.

We then stratified patients into a low ($n = 37$) and a high ($n = 16$) MALAT1 expression group with an established cut-off value (0.432) by using an ROC curve analysis to distinguish the responding and nonresponding patients (Supplementary Fig. S1). The diagnostic sensitivity and specificity reached 89.29% and 52% with the established cut-offs, respectively. In the validation group containing 168 serum samples of colorectal cancer patients, the proportion of patients not responding to chemotherapy was significantly higher in the high MALAT1 expression group than in the low expression group (Fig. 1G). The diagnostic sensitivity and specificity were 75.0% (72/96) and 61.1% (44/72), and the corresponding positive and negative predictive values were 0.72 (72/100) and 0.65 (44/68). In addition, more patients experienced distant metastasis in the high MALAT1 expression group (38 vs. 16). Furthermore, the Kaplan–Meier survival analysis indicated that high MALAT1 expression was associated with shorter OS and DFS in colorectal cancer patients (Fig. 1H).

MALAT1 negatively regulates E-cadherin expression in colorectal cancer cells

Consistent with previous study (3), our established HT29 OxR cells exhibit morphologic and molecular changes consistent with EMT (shown in Supplementary Results and

Supplementary Fig. S2). To define functional links between MALAT1 and EMT, we examined the effects of MALAT1 knockdown on E-cadherin expression. Firstly, we assayed the expression level of MALAT1 and E-cadherin mRNA in colorectal cancer cells. As shown in Fig. 2A, both MALAT1 and E-Cadherin are highly expressed in both noninvasive and invasive colorectal cancer lines, versus normal colon epithelial cells. More importantly, the MALAT1 levels were relatively higher with concurrent low levels of E-cadherin in the invasive cell lines (SW620, LoVo, and HT29 OxR cells) compared with those in the noninvasive cell lines (HCT116, SW480, and HT29 parental cells). It should be pointed out that significantly higher MALAT1 with lower E-cadherin expression was found in HT29 OxR cells compared with HT29 parental cells (Fig. 2A, $P < 0.05$). A significant negative correlation was also observed between the E-cadherin mRNA levels and the MALAT1 expression levels in primary colorectal cancer tissues ($r = -0.4792$, $P < 0.001$; Fig. 2B). We then determined whether MALAT1 regulates E-cadherin expression in colorectal cancer cells. As shown in Fig. 2C, the knockdown effect was best using si-MALAT1 (NO.3) compared with si-MALAT1 (NO.1) and si-MALAT1 (NO.2). Thus, we choose si-MALAT1 (NO.3) for further experiments. MALAT1 knockdown significantly increased E-cadherin expression at both the transcript and protein levels in HT29 parental and OxR cells (Fig. 2D and E). More importantly, MALAT1 knockdown also impaired the migratory and invasive ability of both cell lines (Fig. 2F).

EZH2 is highly expressed and interacts with the 3′ end region of MALAT1 in colorectal cancer

Various reports have indicated that the interaction between MALAT1 and EZH2 might be important for this study (13–14, 22). To verify the potential pathway, we first determined EZH2 expression in colorectal cancer tissues. Results showed that EZH2 mRNA expression was significantly higher in colorectal cancer tissues compared with normal colon tissues (Fig. 3A). Then, we compared the mRNA expression levels for both MALAT1 and EZH2 in colorectal cancer tissues and found a significant positive correlation between the two groups ($r = 0.6105$, Fig. 3B). Furthermore, colorectal cancer patients were divided into two groups using a median EZH2 value of 0.0115, and the association analysis of EZH2 with clinicopathologic parameters indicated that the number of patients expressing high EZH2 was significantly larger in pT (pT3+pT4) and pN (+) patients compared with those in pT1+pT2 and pN (−) patients (Supplementary Table S3). OS was also significantly shorter in the high EZH2 expression group compared with the low EZH2 group (Fig. 3C).

Subsequently, RIP assay was performed with an antibody against EZH2 from nuclear extracts of colorectal cancer cells. As MALAT1 contains more than 8,000 bps, EZH2 protein can only precipitate the MALAT1 fragments that are responsible for the interaction. To achieve a more explicit understanding, 6 MALAT1 fragments (M1–M6), covering the whole sequence from 5′end to 3′end, were separately detected by RT-qPCR with an independent set of primers (Fig. 3D and Supplementary Table S1). The RIP results showed a significant enrichment of MALAT1 with EZH2 antibody compared with the nonspecific IgG antibody (Fig. 3E and Supplementary Fig. S3). Moreover, the region containing the nucleotides 7501 to 8708 at the 3′ end of MALAT1 interacted most strongly with EZH2 (Fig. 3E), but there was no enrichment of β-actin or lncRNA control (Fig. 3F). These data indicate that MALAT1 directly interacts with the EZH2, and EZH2 interacts with the 3′end region of MALAT1.

MALAT1 represses E-cadherin expression by associating with EZH2

To check whether E-cadherin expression is controlled by EZH2, we analyzed the E-cadherin expression level after EZH2 knockdown. EZH2 mRNA and protein expression levels were sufficiently suppressed by si-EZH2 transfection in colorectal cancer cells (Supplementary Fig. S4). EZH2 knockdown significantly increased E-cadherin mRNA and protein levels in colorectal cancer cells (Fig. 4A and B). si-EZH2 significantly impaired cell-migratory and -invasive capacity in both HT29 parental and OxR cells (Fig. 4C). Moreover, the percentage of decreased invasive cells on HT29 OxR cells was 63.2%, which is much higher than that of HT29 parental cells (43.9%, $P < 0.05$). This may suggest that HT29 OxR cells are more sensitive to si-EZH2 compared with HT29 parental cells.

To further address how MALAT1 is involved in EMT through the enrichment of EZH2, we used ChIP analysis to determine the effect of si-MALAT1 on histone modification in the E-Cadherin gene promoter with an anti–H3K27-me3 antibody in SW620 cells. The histone-associated DNAs that were immunoprecipitated with antibody against EZH2 and H3K27-me3 were individually amplified with primer sets covering the E-Cadherin gene promoter regions (Fig. 4D). The binding level of EZH2 and H3K27-me3 was significantly decreased by MALAT1 knockdown compared with control cells (Fig. 4E and F), whereas binding of IgG with E-cadherin promoter showed no significant change after MALAT1 inhibition (Fig. 4G), suggesting that the interaction of EZH2 and H3K27-me3 in the E-cadherin promoter is specifically mediated by MALAT1.

MALAT1 knockdown inhibits oxaliplatin-induced EMT and reverses oxaliplatin resistance through EZH2 in colorectal cancer

Based on the above results, we attempted to demonstrate the biological consequences of MALAT1 on oxaliplatin-induced EMT in a more direct way. Firstly, we evaluated the effect of oxaliplatin on E-cadherin levels in colorectal cancer cells. SW480 and SW620 cells were incubated without oxaliplatin or with a concentration of 0.3 μmol/L oxaliplatin for 48 hours. As expected, E-cadherin mRNA expression was significantly suppressed in colorectal cancer cells incubated with oxaliplatin compared with oxaliplatin-free cells (Fig. 5A). E-cadherin protein levels were also down-regulated after oxaliplatin treatment (Fig. 5B). At the same time, the silencing oligonucleotides or negative control were transfected into another independent group of SW480 and SW620 cells, and then the cells were incubated with 0.3 μmol/L oxaliplatin for 48 hours. The functional role of the MALAT1-EZH2 pathway in the oxaliplatin-induced EMT process was then examined. As shown in Fig. 5C, the suppression of E-cadherin mRNA induced by oxaliplatin was potently relieved by MALAT1 silencing compared with negative controls. Similar results were also found when EZH2 was silenced (Fig. 5D). MALAT1 and EZH2 silencing reversed the oxaliplatin-induced expression change of E-cadherin and Vimentin at protein levels (Fig. 5E). Moreover, the *in situ* expression of E-cadherin was detected by immunofluorescence in HT29 cells as described in Materials and Methods. Decreased E-cadherin expression was observed after being treated with oxaliplatin compared with oxaliplatin-free cells; however, this

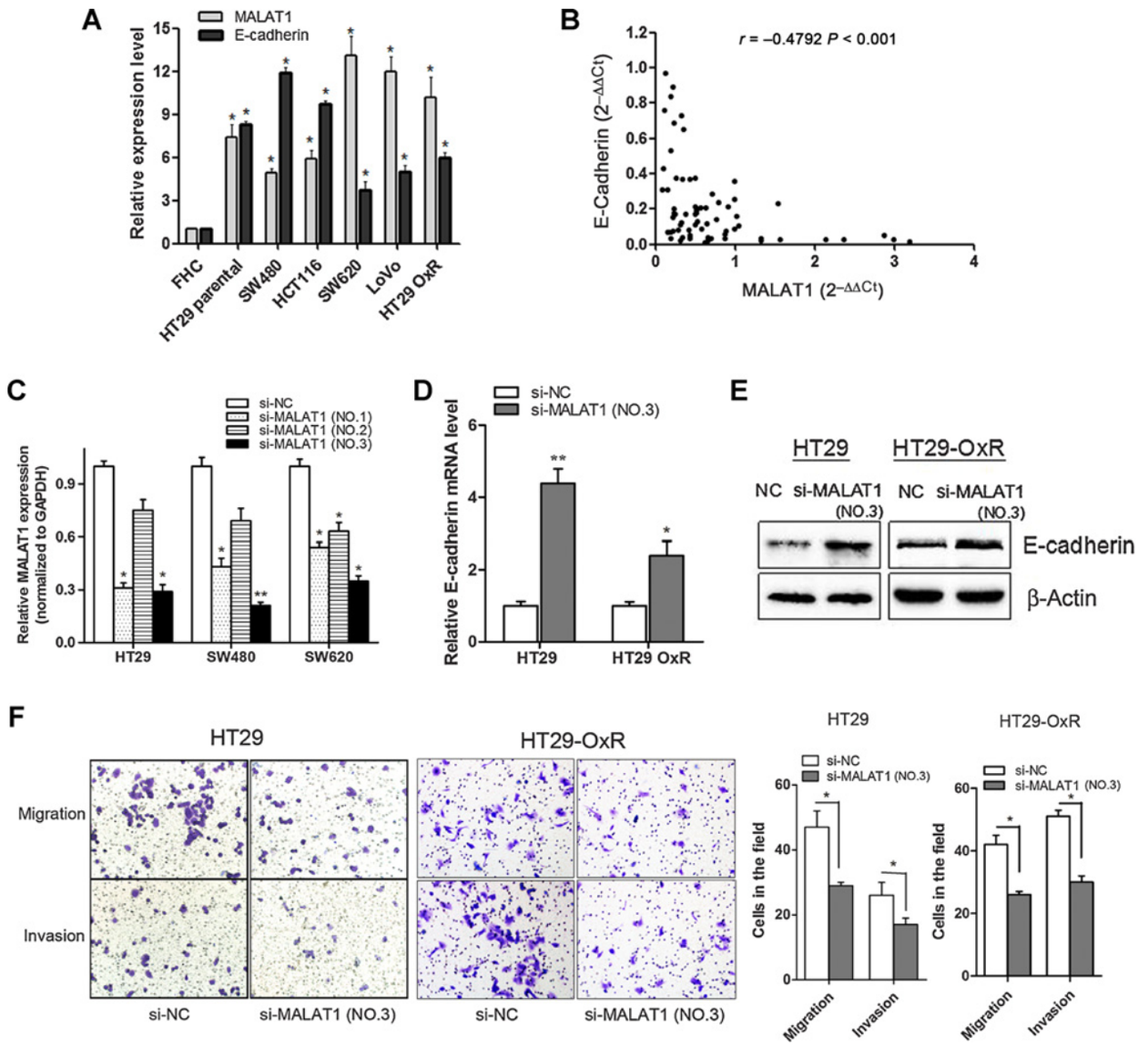

Figure 2.

MALAT1 negatively regulates E-cadherin expression in colorectal cancer cells. **A,** MALAT1 and E-cadherin mRNA levels were evaluated by RT-PCR in three noninvasive (HCT116, SW480, and HT29 parental cell lines) and three invasive colorectal cancer cell lines (SW620, LoVo, and HT29 OxR cell lines). FHC cells were used as the control; *, $P < 0.05$. **B,** Negative correlation between the E-cadherin mRNA levels and the MALAT1 levels in 68 colorectal cancer tissue samples ($r = -0.4792$, $P < 0.001$). **C,** Colorectal cancer cells were transfected with 100 nmol/L of the indicated siRNA. Forty-eight hours after transfection, RT-qPCR was performed using the primers for MALAT1. *, $P < 0.05$ vs. si-NC. **D,** E-cadherin mRNA levels in colorectal cancer cells were detected using RT-qPCR after transfection of si-MALAT1 and si-NC. *, $P < 0.05$ versus si-NC. **E,** Western blot analysis of E-cadherin after si-MALAT1 treatment for 72 hours in HT29 parental and OxR cells. **F,** HT29 parental and OxR cells were transfected with 100 nmol/L of si-MALAT1 for 48 hours. Migration and invasion assay was performed as described in Material and Methods. *, $P < 0.05$.

downregulation of E-cadherin was significantly reversed by MALAT1 or EZH2 knockdown (Fig. 5F). Transwell assay also validated that MALAT1 or EZH2 knockdown impaired the oxaliplatin-induced invasion ability of colorectal cancer cells (Fig. 5G and Supplementary Fig. S5). Finally, we investigated whether MALAT1 contributes to oxaliplatin resistance through interacting with EZH2. As expected, MALAT1 or EZH2 knockdown promoted the oxaliplatin-induced cell cytotoxicity in HT29 OxR cells (Fig. 5H), and the concentration-effect curve indicated that the IC_{50} value of oxaliplatin for HT29 OxR cells transfected with si-MALAT1 (6.12 μmol/L) or si-EZH2 (4.98 μmol/L) was much lower than that for HT29 OxR cells transfected with si-NC (10.2 μmol/L; Fig. 5I). Taken together, our results showed that the MALAT1-EZH2 regulatory pathway plays a crucial role in oxaliplatin-induced EMT and chemoresistance in colorectal cancer.

MALAT1 interacts with miR-218, which is associated with chemoresponse to FOLFOX treatment in colorectal cancer

Using *miRcode* (http://www.mircode.org/mircode), a computer algorithm to identify miRNA target genes, including lncRNAs

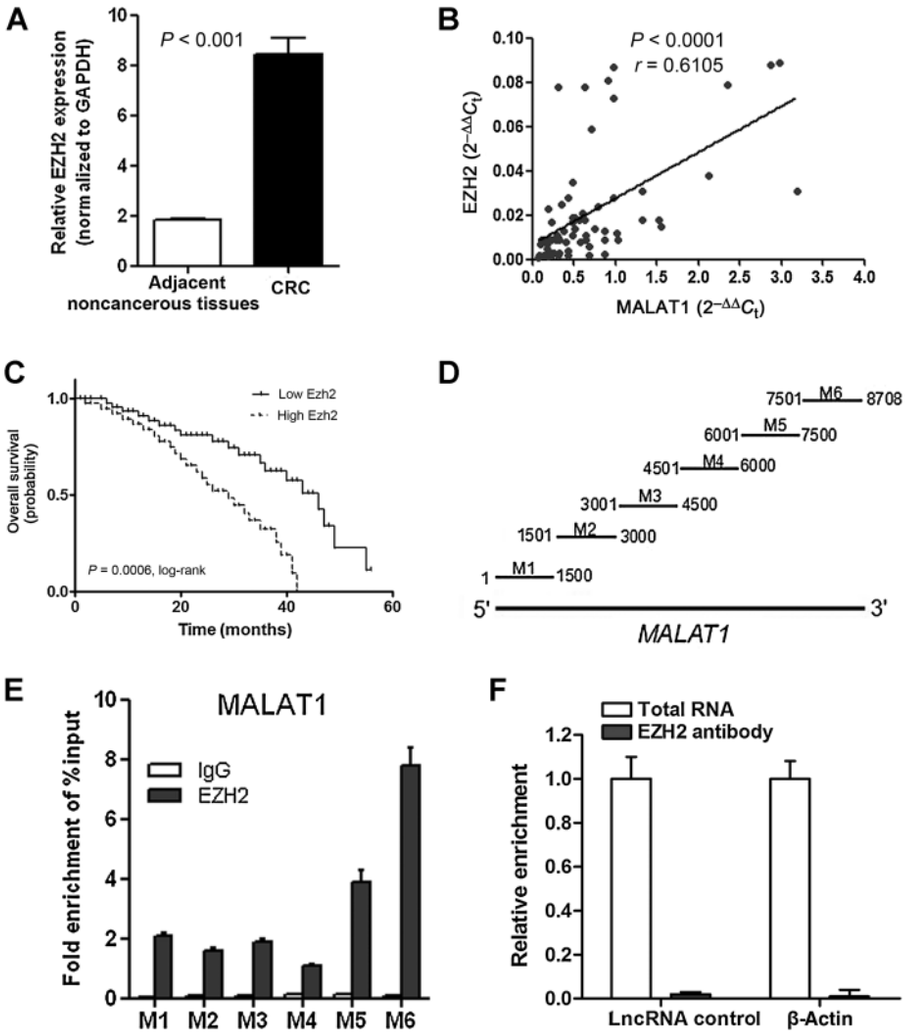

Figure 3.
EZH2 is significantly increased in colorectal cancer and interacts with the 3′ end of MALAT1. **A,** RT-qPCR was used to compare the EZH2 mRNA expression in 68 colorectal cancer tissues and primary adjacent noncancerous tissues. **B,** Positive correlation was found between the E-cadherin mRNA levels and the MALAT levels in 68 colorectal cancer tissue samples by using the Spearman test ($r = 0.6105$, $P < 0.001$). **C,** Sixty-eight colorectal cancer patients were divided into two groups using a median EZH2 value of 0.0115, and the association of MALAT1 expression with OS was analyzed with a Kaplan–Meier plot. **D,** Schematic diagram of MALAT1 regions M1–M6 separately determined by RT-qPCR using an independent set of primers. **E,** RIP experiments were performed using the EZH2 antibody to immunoprecipitate RNA and an independent set of primers to detect different regions of MALAT1. **F,** RIP experiments were performed using an EZH2 antibody to immunoprecipitate RNA and primers to detect lncRNA control and β-actin.

(23), we identified an miR-218 binding site on MALAT1 (Fig. 6A). We have previously demonstrated that miR-218 is a tumor-suppressor gene and may be a novel chemotherapeutic indicator and target for 5-FU in colorectal cancer (2); thus, we sought to explore the potential interaction between MALAT1 and miR-218. Although the expression of MALAT1 was high in colorectal cancer cell lines compared with normal colon cells, miR-218 expression was significantly lower in colorectal cancer cell lines (Fig. 6B). The Spearman test indicated a significant negative correlation between MALAT1 and miR-218 expression in 46 colorectal cancer tissues (Fig. 6C). Moreover, knockdown of MALAT1 significantly increased miR-218 expression in colorectal cancer cells (Fig. 6D); however, after overexpression of miR-218, MALAT1 expression was significantly decreased (Fig. 6E). These results suggest that the interaction between MALAT1 and miRNA-218 has reciprocal effects.

As both MALAT1 and miR-218 play critical roles during chemotherapy, we sought to investigate the prognostic significance of the interaction between the MALAT1 and miR-218 in colorectal cancer patients who received standard FOLFOX (oxaliplatin combined with 5-FU and leucovorin) treatment (19 response and 27 nonresponse), and ROC curve analysis was performed to establish the optimal cut-off value of MALAT1 (0.378) and miR-218 (0.005) for distinguishing the responding and nonresponding patients (Supplementary Fig. S6). Patients were stratified into high/low MALAT1 and miR-218 expressing groups under this cut-off. The number of patients not responding to chemotherapy was significantly higher in the high MALAT1 and low miR-218 expression group (Fig. 6F). More importantly, the Kaplan–Meier survival analysis showed that patients with low MALAT1 and high miR-218 expression had the longest OS, whereas the high MALAT1 and low miR-218 group showed the shortest OS (Fig. 6G). This indicates that the biomarker panel of MALAT1 combined with miR-218 could be a potential novel chemotherapeutic indicator for colorectal cancer patients receiving standard FOLFOX treatment.

On the basis of our results, a model was proposed for the potential role of MALAT1 in oxaliplatin-induced EMT in colorectal cancer (Fig. 6H). For patients with high MALAT1 expression (left), MALAT1 tethers EZH2 to *CDH1* promoter and suppresses miR-218 during oxaliplatin treatment, which finally promotes colorectal cancer cell EMT, metastasis, and chemoresistance; for patients with low MALAT1 expression (right), MALAT1 fails to suppress the expression of E-cadherin and

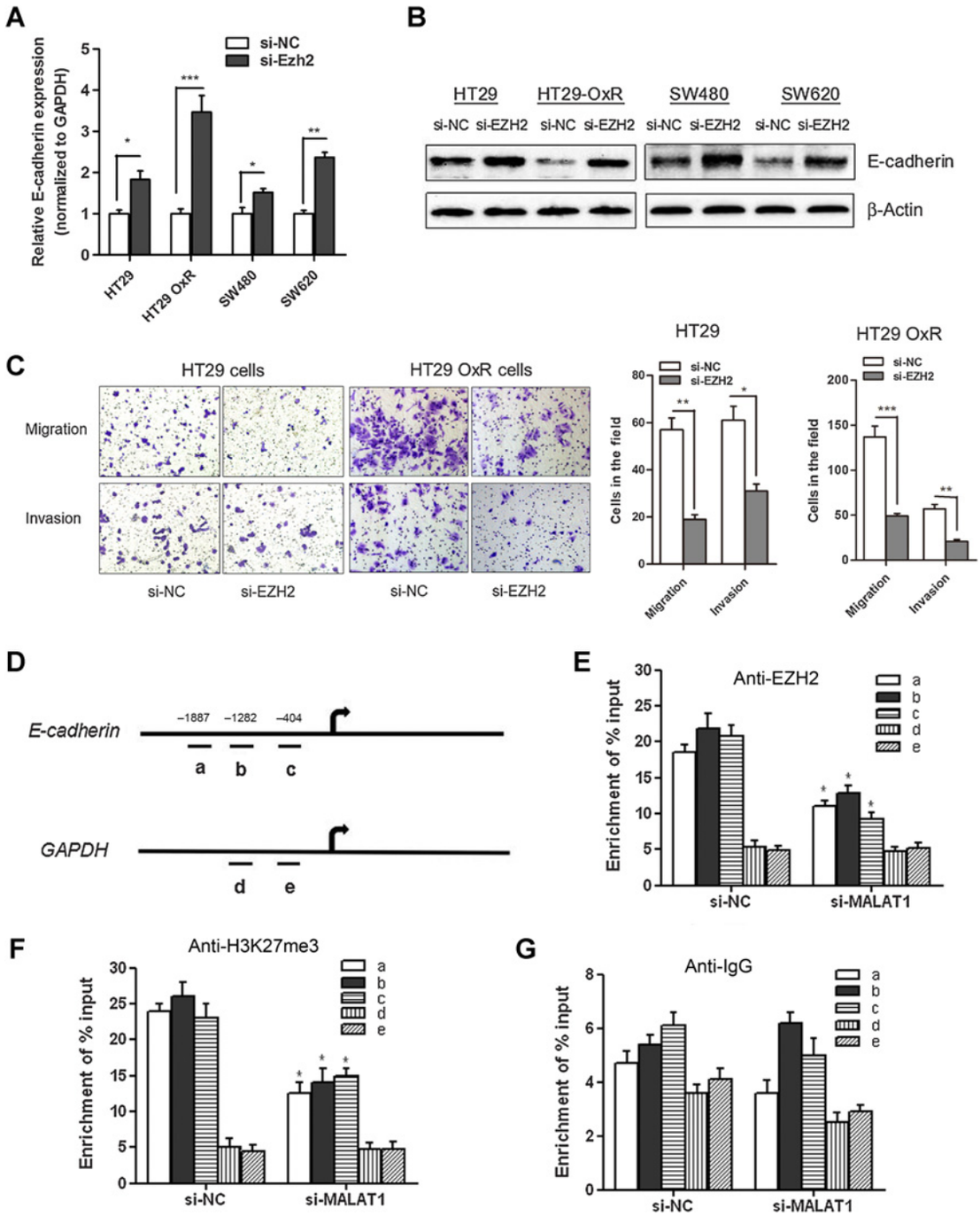

Figure 4.

Knockdown of MALAT1 increases E-cadherin expression by associating with EZH2. **A** and **B,** E-cadherin mRNA (*CDH1*) level (**A**) and E-cadherin protein level (**B**) were determined in colorectal cancer cells after si-EZH2 treatment for 48 hours. **C,** HT29 parental and OxR cells were transfected with si-EZH2 for 48 hours. Then, the migration and invasion assay was performed as described in Materials and Methods. **D–G,** ChIP analysis of SW620 treated with si-MALAT1 was conducted on E-cadherin promoter (primer a-c) and GAPDH promoter (primer d-e) regions using the indicated antibodies. Enrichment was determined relative to input controls. These results show data from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

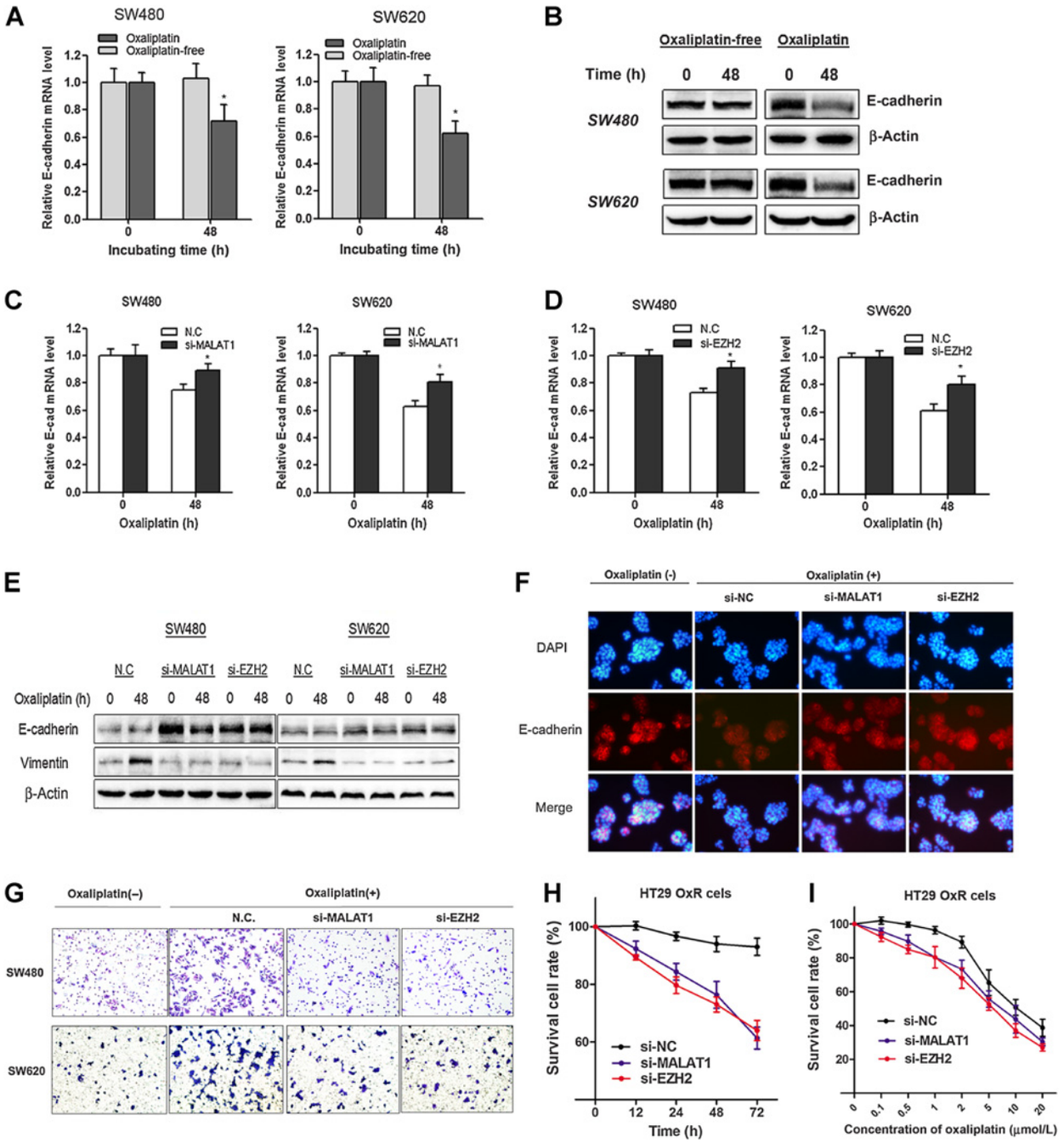

Figure 5.

MALAT1 knockdown inhibits oxaliplatin-induced EMT through EZH2 in colorectal cancer. **A,** SW480 and SW620 cells were incubated without oxaliplatin or with a concentration of 0.3 μmol/L oxaliplatin for 48 hours. Then, RT-qPCR was performed to detect the E-cadherin mRNA expression at the 0-hour and 48-hour point. *, $P < 0.05$ versus oxaliplatin-free cells at 48 hours. **B,** Western blot was performed to detect the expression of E-cadherin protein after being treated with or without oxaliplatin. **C** and **D,** After transfection of si-MALAT1 (**C**) or si-EZH2 (**D**) for 48 hours, the E-cadherin mRNA levels were detected by RT-qPCR in colorectal cancer cells incubated with culture medium containing oxaliplatin. *, $P < 0.05$ versus NC at 48 hours. **E,** E-cadherin and Vimentin protein expression levels were determined using the Western blot method after si-MALAT1 or si-EZH2 treatment for 48 hours in SW480 and SW620 cells. **F,** The immunofluorescence of E-cadherin–stained sections followed by counterstaining with DAPI in HT29 cells (40×). **G,** SW480 and SW620 cells were transfected with 100 nmol/L of the indicated siRNA. Invasion assay was performed as described in Materials and Methods. *, $P < 0.05$. **H,** Cell viability assay showed that MALAT1 or EZH2 knockdown promoted the oxaliplatin-induced cell cytotoxicity in HT29 OxR cells. **I,** The concentration-effect curve indicated that the IC_{50} value of oxaliplatin for HT29 OxR cells transfected with si-MALAT1 (6.12 μmol/L) or si-EZH2 (4.98 μmol/L) was much lower than that for HT29 OxR cells transfected with si-NC (10.2 μmol/L). These results show data from at least three independent experiments.

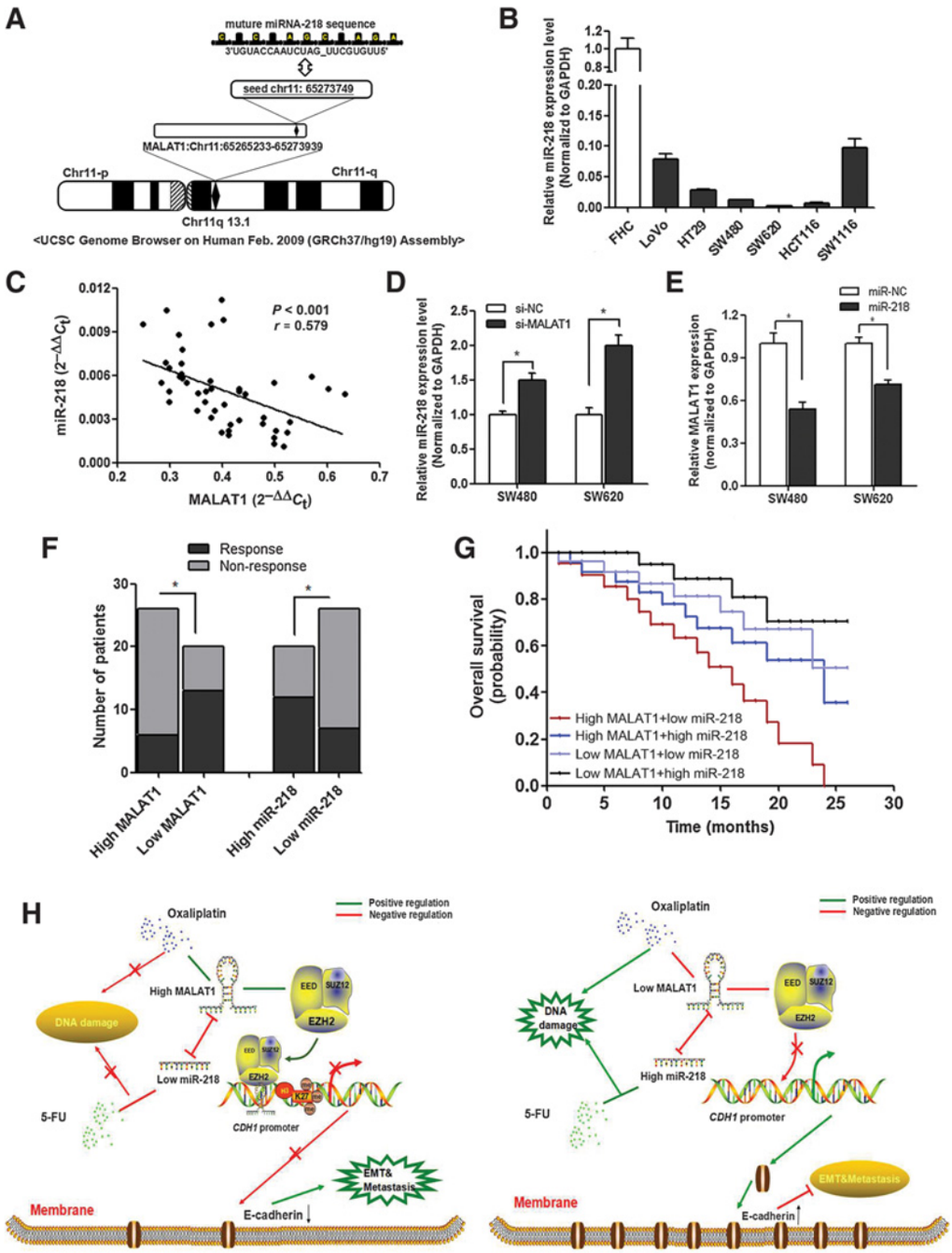

Figure 6.

The interaction between MALAT1 and miR-218 is associated with chemoresponse to FOLFOX treatment in colorectal cancer patients. **A,** Representation of the miR-218 binding site in MALAT1 based on *miRcode* (http://www.mircode.org/mircode/). **B,** RT-qPCR was used to compare the expression of miR-218 in seven colorectal cancer cell lines and normal colon cell line FHC. **C,** Negative correlation was found between the miR-218 levels and the MALAT levels in 46 colorectal cancer tissue samples by using Spearman test ($r = 0.5790$, $P < 0.001$). **D,** Relative miR-218 expression was determined by RT-qPCR in SW480 and SW620 cells after transfection with si-MALAT1. **E,** MALAT1 expression in SW480 and SW620 cells was detected after transfection with miR-218 mimics. **F,** Forty-six tissue samples from colorectal cancer patients were divided into a low and high MALAT1-expressing group by establishing a ROC curve, then, the number of patients that respond or not respond to chemotherapy in the high and low MALAT1 (miR-218) expressing group was analyzed ($P < 0.05$, Fisher exact test). **G,** The OS curves for colorectal cancer patients receiving standard FOLFOX treatment in different groups according to MALAT1 and miR-218 expression (log-rank test). *, $P < 0.05$. **H,** Schematic representation of the consequence of MALAT1 on colorectal cancer patients receiving oxaliplatin-based treatment. For patients with high MALAT1 expression (left), MALAT1 tethers EZH2 to *CDH1* promoter and suppresses miR-218 during oxaliplatin treatment, which finally promotes colorectal cancer cell EMT, metastasis, and chemoresistance; for patients with low MALAT1 expression (right), MALAT1 fails to suppress the expression of E-cadherin and miR-218, which inhibits colorectal cancer cell EMT and finally promotes chemosensitivity.

miR-218, which inhibits colorectal cancer cell EMT and finally promotes chemosensitivity.

Discussion

Despite recent chemotherapeutic regimens that have significantly increased survival in metastatic disease, nearly all colorectal cancer patients finally become chemoresistant accompanied with distant metastasis (5). Understanding the mechanism of resistance in colorectal cancer is essential to optimizing current therapeutic strategies (24). LncRNA MALAT1 was first recognized as a marker for metastasis development in the early stages of lung adenocarcinoma (10) and, more recently, in colorectal cancer (15, 25, 26). It has been shown to play an important role in diverse biological processes, such as development, tumorigenesis, and the metastatic cascade (13), but its function during the process of chemotherapy failure remains unknown. Previous study has shown that MALAT1 was overexpressed in colorectal cancer and was a biomarker of poor prognosis (27). By using the RT-qPCR-D method that we have previously established (20, 28), we validated the upregulation of MALAT1 and demonstrated that high MALAT1 expression was negatively associated with chemotherapy response and positively associated with distant metastasis in colorectal cancer patients receiving oxaliplatin-based chemotherapy. This is also of considerable therapeutic significance because of the importance of discovering a MALAT1 inhibition method that may provide a new modulation strategy to overcome chemoresistance.

We attempted to unravel the molecular switch of MALAT1 controlling this malignant phenotype and elucidate the underlying mechanisms on the metastatic evolution of colorectal cancer. An OxR HT29 OxR cell line was established using the method previously reported (3). As expected, the OxR cells were resistant to oxaliplatin and shown to undergo EMT, which had a tight link with cancer metastasis. Moreover, the MALAT1 expression in HT29 OxR cells was significantly higher than in HT29 parental cells, and the mesenchymal-like change was reversed after MALAT1 knockdown in HT29 OxR and parental cells. Furthermore, MALAT1 expression was negatively correlated with E-cadherin in colorectal cancer cells and specimens. After siRNA knockdown of MALAT1, E-cadherin mRNA and protein expression were significantly suppressed in both HT29 OxR and parental cells. These results were consistent with previous reports, which showed that MALAT1 functions as an oncogene in colorectal cancer (15, 25, 26), and further indicated that MALAT1 may participate in the oxaliplatin-induced EMT process by negatively regulating E-cadherin expression in colorectal cancer.

Human genome sequence data indicate that more than 90% of the DNA sequence is actively transcribed, but only 2% of it encodes protein (29). Thus, considerable quantities of lncRNAs are activated transcriptionally. HOTAIR has been proven to target EZH2 to govern the cells' epigenetic state and subsequent gene expression (30). Previous reports have shown that EZH2 is highly expressed in many cancers, promoting cancer progression (13, 18–19). To determine the underlying molecular mechanisms by which MALAT1 regulated downstream effectors in colorectal cancer, we focused on EZH2. Similar to previous study (18), we demonstrated that EZH2 mRNA expression was significantly higher in colorectal cancer patients and was associated with higher stage and shorter OS. There was a significant positive correlation between MALAT1 and EZH2 mRNA expression. Furthermore, our RIP results showed that MALAT1 specifically interacted with EZH2, and EZH2 was mostly associated with the 3′end of the MALAT1 region, which provided a new direction for studies of the biological function of MALAT1.

It is known that EZH2 enhances methylation of H3K27, leading to gene silencing involved in cancer progression and metastasis (17). Downregulation of tumor suppressor gene E-Cadherin (*CDH1*) is typically observed during EMT in colorectal cancer (31); thus, we sought to investigate whether the recruited EZH2 triggered suppression of E-cadherin in oxaliplatin-induced EMT. After silence of EZH2, both E-cadherin mRNA and protein expression were significantly increased in colorectal cancer cells; whereas the migratory and invasive capacity was suppressed in both HT29 OxR and parental cells. HT29 OxR cells are more sensitive to si-EZH2 compared with HT29 parental cells, which further verified our hypothesis about the role of EZH2 in oxaliplatin-induced EMT. The ChIP assay further validated that MALAT1 decreases E-cadherin expression through EZH2-mediating H3K27-me3 formation in colorectal cancer. Furthermore, colorectal cancer cells experienced EMT and attained increased invasive ability after being treated with oxaliplatin; this increased mesenchymal-like change was partially suppressed by MALAT1 or EZH2 knockdown. This is further validated by the immunofluorescence results. In addition, MALAT1 or EZH2 knockdown promoted the oxaliplatin-induced cell cytotoxicity and reduced the IC_{50} value of oxaliplatin. Ultimately, we demonstrated that oxaliplatin induced chemoresistance and EMT in colorectal cancer cells by the MALAT1/EZH2 pathway.

Finally, we focused on the interaction between MALAT1 and miRNAs, which have been reported to play important roles in tumorigenesis, metastasis, and drug resistance in various malignancies (9, 32). As a newly described regulatory mechanism, lncRNA can influence the regulation of miRNA generation and interfere with miRNA pathways as a natural miRNA sponge (33–35). Thus, lncRNA may modulate derepression of miRNA's target gene expression. Various examples in the literature have demonstrated that miR-218 is a tumor-suppressor gene and could significantly suppress the EMT process (36, 37). We previously revealed that miR-218 promoted cell apoptosis and enhanced 5-FU–based chemosensitivity in colorectal cancer cells by targeting BIRC5, a key member of the inhibitors of apoptosis gene (*IAP*) family (2). *MiRcode* analysis showed that there is an miR-218 MALAT1 binding site, and our results validated the interaction between MALAT1 and miR-218. More importantly, patients with low MALAT1 and high miR-218 expression had a better survival rate compared with high MALAT1 and low miR-218 expression patients receiving standard FOLFOX treatment. This is far more important in view of the fact that the FOLFOX regimen is the first-line therapy for metastatic colorectal cancer (38, 39), and the combination of MALAT1 and miR-218 could be a potential prognostic and therapeutic target.

In conclusion, the present work has identified that lncRNA MALAT1 was correlated with tumor metastasis and associated with poor response to oxaliplatin-based chemotherapy in colorectal cancer patients. MALAT1 mediates oxaliplatin-induced EMT through EZH2 and interacts with miR-218. These indicated that overexpression of MALAT1 confers a potently poor therapeutic efficacy. Thus, MALAT1 may be a potential functional biomarker and therapeutic target in colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Li, X. Zhang, L. Wang, C. Wang
Development of methodology: P. Li, Y. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Li, H. Wang, C. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Li, T. Liu, L. Du, C. Wang
Writing, review, and/or revision of the manuscript: P. Li, X. Zhang, Y. Yang, C. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Li, L. Du, C. Wang
Study supervision: C. Wang

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