Aberrant MCM10 SUMOylation induces genomic instability mediated by a genetic variant associated with survival of esophageal squamous cell carcinoma

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Graphical Abstract

1. A two-stage survival study consisting of 1407 subjects finds a significant association between MCM10-rs2274110 and ESCC survival.
2. The MCM10 variant can increase MCM10 SUMOylation levels and result in MCM10 aberrant overexpression, which facilitates ESCC cells proliferation and migration by inducing genomic instability.
3. The MCM10 inhibitors Suramin and its analogues can block ESCC cells metastasis.
Aberrant MCM10 SUMOylation induces genomic instability mediated by a genetic variant associated with survival of esophageal squamous cell carcinoma

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Abstract

Background: Esophageal squamous cell carcinoma (ESCC) is one of the common gastrointestinal malignancy with an inferior prognosis outcome. DNA replication licensing aberration induced by dysregulation of minichromosome maintenance proteins (MCMs) causes genomic instability and cancer metastasis. SUMOylation modification plays a pivotal role in regulation of genomic integrity, while its dysregulation fueled by preexisting germline variants in cancers remains poorly understood.

Methods: Firstly, we conducted two-stage survival analysis consisting of an exome-wide association study in 904 ESCC samples and another independent 503 ESCC samples. Then, multipronged functional experiments were performed to illuminate the potential biological mechanisms underlying the promising variants, and MCM10 influences the ESCC progression. Finally, we tested the effects of MCM10 inhibitors on ESCC cells.

Results: A germline variant rs2274110 located at the exon 15 of MCM10 was identified to be significantly associated with the prognosis of ESCC patients. Individuals carrying rs2274110-AA genotypes confer a poor survival (hazard ratio = 1.61, 95% confidence interval = 1.35–1.93, p = 1.35 × 10−7), compared with subjects...
carrying rs2274110-AG/GG genotypes. Furthermore, we interestingly found that the variant can increase SUMOylation levels at K669 site (Lys[K]699Arg[R]) of MCM10 protein mediated by SUMO2/3 enzymes, which resulted in an aberrant overexpression of MCM10. Mechanistically, aberrant overexpression of MCM10 facilitated the proliferation and metastasis abilities of ESCC cells in vitro and in vivo by inducing DNA over-replication and genomic instability, providing functional evidence to support our population finding that high expression of MCM10 is extensively presented in tumor tissues of ESCC and correlated with inferior survival outcomes of multiple cancer types, including ESCC. Finally, MCM10 inhibitors Suramin and its analogues were revealed to effectively block the metastasis of ESCC cells.

**Conclusions:** These findings not only demonstrate a potential biological mechanism between aberrant SUMOylation, genomic instability and cancer metastasis, but also provide a promising biomarker aiding in stratifying ESCC individuals with different prognosis, as well as a potential therapeutic target MCM10.

**KEYWORDS**
ESCC, genomic instability, MCM10, SUMOylation, survival

### 1 | INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the common gastrointestinal malignancy in China, and its clinical prognosis is poor with 5-year survival < 30%. Surgery supplemented with chemotherapy or chemoradiotherapy was the standard treatment of ESCC. However, these treatments were accompanied by a considerable increase in health-related quality of life and yet still a poor prognosis. Accordingly, effective prognostic markers or therapy targets need to be identified for this malignant cancer. Inherited genetic polymorphisms have been proved to influence their phenotypic differences, such as genetic susceptibility of disease, treatment responses, drug resistance, and clinical prognosis. Previously a genome-wide association study (GWAS) has identified a regulatory variant in SLC39A6 that may serve as a prognostic marker of ESCC. These findings indicate that preexisting germline variants are worthy to be investigated for identifying both effective prognostic markers and therapy targets for this cancer.

Genomic instability, a hallmark of human cancer, is the major driver of tumor evolution and also shown to be associated with poor clinical prognosis, metastasis, and therapeutic resistance of tumors. The proper licensing of DNA replication origins is an essential event for timely duplication and stability of genome. This replication licensing system requires the stable loading of some replication licensing factors, such as origin recognition complex (ORC), chromatin licensing and DNA replication factor 1 (CDT1), and the recruitment of the minichromosome maintenance (MCM) complex at replication origins. Errors or aberration in replication licensing system might result in genomic instability across evolution and cell development. It was reported that in normal cells, the licensing system of DNA replication usually protect cells from replicative stress and genomic instability. However, in tumor cells, these factors were shown to be widely upregulated and were associated with cancer progression or poor clinical outcomes. Depletion of the MCMs inhibited the tumor growth and progression, suggesting they may not only be prognostic markers, but play important roles in cancer development. It was also reported that in pre-malignant cells, the overexpression of replication licensing factors, such as CDC6 and CDT1, could facilitate malignant behavior conversion. Besides, the combined deregulation of CDC6 and CDT1 was also shown to lead to DNA re-replication in progenitor cells and lethal tissue dysplasia. Therefore, these accumulating evidences provide a conjecture supporting that overexpression of these replication licensing factors, including MCMs families, in human cancer cells may cause DNA “over-replication” stress and genomic instability, which might further deteriorate cancer progression and poor prognosis.

SUMOylation is one of post-translational protein modification (PTM) and recognized by small ubiquitin-like modifier (SUMO) and plays important roles in responding cellular stress, stabilizing genomic integrity, regulating transcriptional patterns of genes, and signal transduction pathways and thus which is essential for cell homeostasis in eukaryotes. Importantly, SUMO pathway
components appear to be upregulated in many cancers. Tumor cells may indeed require this “SUMOylation activation” to maintain the compromised robustness or otherwise easily occur dysregulation of gene transcriptional expression patterns and pathological activation of cancer signaling pathways. Thus, in a potentially hostile microenvironment, SUMOylation would contribute substantially to facilitating proliferation and migration of cancer cells. Given that SUMOylation-regulated mechanisms are essential for cells in mammals, loss-of-function caused by germline variants in recognition sites of the SUMOylation process might be a potential biological mechanism contributing to diseases development and prognosis.

Here, we firstly conducted a two-stage survival analysis totally consisting of 1407 ESCC samples by using our previous exome-chip data and Taqman genotyping. We identified a SUMOylation variant rs2274110 located at the 15th exon of MCM10, which confers an inferior prognosis of ESCC. Mechanistically, we found that this variant upregulates the expression of MCM10 protein by increasing its SUMOylation level mediated by SUMO2/3. Furthermore, we indicated that upregulation of MCM10 facilitates the metastasis of ESCC cells both by inducing genomic instability, which may be induced by the DNA over-replication. Moreover, we also showed that the MCM10 inhibitors Suramin and its analogues (NF157, NF546, and PPADS) can be used as potential anti-cancer agents for ESCC, but are warranted to deconstruct the precise mechanisms and effective targets of these drugs before its clinical use. Our data provide insights into the link between aberrant SUMOylation, genomic instability, and cancer.

2 METHODS

2.1 Subjects and variants genotyping

In the present study, a two-stage survival analysis totally consisting of 1407 ESCC samples was performed, and the baseline characteristics of all samples were detailed in Table S1. The variants genotypes of samples were detecting by using the Illumina HumanExome Beadchip system in the discovery stage, which has been detailed described previously. Briefly, we enrolled a total of 904 ESCC patients in the discovery stage who had available genotype data of variants, detailed survival time and outcomes, smoking and drinking status and tumor stage, from the Cancer Hospital, Chinese Academy of Medical Sciences in Beijing, China. In addition, a total of 503 ESCC patients who were enrolled from multiple hospitals in Wuhan were genotyped with Taqman genotyping assay (ABI 7500HT System, Applied Biosystems, USA) in the replication stage. We applied medical records and interviews to collect baseline characteristics and clinical information of ESCC individuals. The survival time of ESCC in this study were determined as the number of days from the date of first diagnosis to the date of death or date of last known contact. Noticeably, written informed consent was collected from all participants, and this study was also approved by the institutional review board of Huazhong University of Science and Technology (HUST, [2016]IEC(S160)).

2.2 Cell lines

ESCC cell lines KYSE30 and KYSE150 obtained from the China Center for Type Culture Collection (Shanghai, China), were cultured with Dulbecco’s modified eagle’s medium, 10% fetal bovine serum and 1% antibiotics consisting of penicillin and streptomycin (Gibco, USA), respectively. Noticeably, KYSE30 and KYSE150 cell lines used here were by authenticated by short tandem repeat profiling (Applied Biosystems, USA) and validated for the absence of mycoplasma contamination (MycoAlert, USA).

2.3 Construction and transfections

For transient transfection assay, the full-length cDNA of MCM10 consisting of rs2274110[A] allele (named as MCM10[A]) or rs2274110[G] allele (named as MCM10[G]) was commercially synthesized and inserted into the pcDNA3.1(+) vector (Invitrogen, USA) by Genewiz (Suzhou, China). For lentivirus production and transfection, the full-length cDNA of MCM10[A] or MCM10[G] was inserted into the pLVX-PGK-Puro and rLV-Luciferase-Hygro vectors. The X-tremeGENE9 reagent (Roche, USA) and Lenti-XTM concentrator were used to produce Lentivirus in 293T cells. Lentivirus-containing plasmids were transfected into ESCC KYSE30 and KYSE150 cells, and 2 mg/ml puromycin was used for antibiotic selection.

2.4 RNA interference and CRISPR/Cas9 system

The siRNAs targeting SUMO2/3 and shRNAs targeting MCM10 used for knockdown assays were synthesized by RiboBio company (Guangzhou, China). All sequences of oligonucleotides were listed in Table S2. The quantitative reverse transcription PCR (qRT-PCR) and western blotting were conducted to evaluate the transfection effect (Figures S2A and S2B). Furthermore, we applied CRISPR-Cas9 editing system (Genloci Biotechnologies, China) to knockout MCM10 in KYSE30 and KYSE150 cell lines with pGK1.1-CRISPR-Cas9 vector (Cat# GP0134). We designed the single guide RNAs (sgRNAs) targeting MCM10 using by
the CRISPR design tool (http://crispr.mit.edu). The sgRNA sequence targeting MCM10 sites are detailed in Table S2, and the effects of MCM10 knockout in ESCC cells were determined by Western-blotting assay (Figure S2C).

2.5 qRT-PCR

TRIzol reagent (Applied Biosystems, USA) was applied to harvest total RNA of ESCC tissues or cell lines. Subsequently, SuperScriptIII First-Strand Synthesis System (Invitrogen, USA) and Power SYBRTM Green PCR Master Mix (Applied Biosystems, USA) was used to conduct reverse transcription and quantitative PCR assays, respectively. Noticeably, the expression of GAPDH was used to normalize the expression of target genes. All specific qPCR primers used can be seen in Table S2.

2.6 Western blotting

Total protein of ESCC tissues or cell lines were extracted by RIPA lysis buffer supplemented with protease inhibitors PMSF (Beyotime, China), and PhosSTOP (Sigma, USA) and then followed by sonication homogenization. Protein was incubated with primary antibodies against SUMO2/3 (1:1,000; Cat# 4971, CST), MCM10 (1:1,000; ab3733, Abcam), and β-actin (1:1,000; Cat# 60008-1-Ig, Proteintech, China) at 4°C over night.

2.7 Co-Immunoprecipitation

KYSE30 and KYSE150 cells were cultured in 15 cm cell plate and firstly transfected with pcDNA-Ctrl, MCM10[A] or MCM10[G] plasmids. After 48 h transfection, ESCC cells were lysed with a RIPA buffer supplement with protease inhibitors. For co-immunoprecipitation (CO-IP) experiments, 10 μg MCM10 antibody (ab3733, Abcam) or SUMO2/3 antibody (Cat # 4971, CST) was incubated with 1 mg cellular protein at 4°C for 3 h. Immunoprecipitates were captured and incubated with Protein A/G beads (20 μL; Cat# LSKMAGAG02, Millipore, USA) at 4°C overnight. Furthermore, the beads were washed extensively by lysis buffer for three times. Subsequently the elution was heated with 4 × loading buffer and were used to analyze by western blotting.

2.8 Liquid chromatography tandem mass spectrometry analysis

For beads samples, the preparation process of protein was adapted based on previously reported research. The wash buffer consisting of 100 mM Tris-Cl and 150 mM NaCl was added and used to wash the IP beads binding with proteins. Furthermore, we added 50 μl of elution buffer consisting of 1% SDC/100 mM Tris-Cl, 10 mM TCEP, and 40 mM CAA to elute protein and followed by reduction and alkylation reaction at 95°C for 10 min. Subsequently, we used the self-made SDB tip columns to desalt the peptide. Finally, the protein samples were dried by vacuum and stored at -80°C until MS measurement. Liquid chromatography tandem mass spectrometry (LC-MS/MS) detection was conducted with hybrid quadrupole-TOF mass spectrometer (TripleTOF 5600+, SCIEX, USA) equipped with a nanoLC system (Eksigent). Detailed LC/MS processes had been described in our previous research. Variable modifications were assigned as SUMO2135 and SUMO3549 of lysine. Only high-quality peptide assignments with more than 95% confidence can be selected for further analysis, and the p < 0.05 was determined as the statistically significant.

2.9 Cell proliferation and migration

For the cell proliferation assay, cells were digested by trypsin, and 100 μl of cell suspension counted 2.5 × 10² cells were seeded in 96-well plate. After incubation for 24, 48, 72, and 96 h, we detected the absorbance at 450 nm to reflect the cell viability using CCK-8 assay kit (Dojindo, Japan). For the cell migration assay, the cells were firstly starved with serum-free medium for 8 h. Then, prior to the cell digestion, mitomycin C (10 μg/ml; Roche, Germany) was added into cells and incubated for 2 h. Cell suspension containing 2.5 × 10³ serum-starved cells were placed and cultured in the upper chamber of Transwell Clear Polyester Membrane Inserts (Cat# 3422, 8-μm pore filter, Corning Costar), while in the bottom chamber were added by culture media containing 20% FBS. After 24 h in culture, the migrated cells were fixed by 3.7% formaldehyde, permeabilized with 100% methanol and stained with 0.5% crystal violet, respectively. Then, the cells on upper surface of the Transwell chamber were scraped by a cotton swab, and the cells bottom surface of the Transwell filter were counted as the invasive cells. The number of migrated cells were examined by microscopy and counted in eight random areas.

2.10 Animal experiments

Female, aged 4–5 weeks BALB/c nude mice were purchased from the Vital River Laboratory Animal Technology (Beijing, China). For the lung metastasis model, we
injected luciferase labeled ESCC cells (0.1 ml, 2 × 10^6 cells) into nude mice via tail vein. Eight weeks after injection, the nude mice were injected with d-luciferin intraperitoneally (100 μl, 30 mg/ml, Promega, USA) and reaction 10 min. Then, the nude mice were maintained under general anesthesia with isoflurane and further imaged by the IVIS system. The quantitation of lung metastases in vivo was measured by using an IVIS Lumina II (Caliper) equipped with Living Image software (PerkinElmer). Finally, we used paraformaldehyde to fix the lung tissues of nude mice and stain it with H&E. All experimental procedures were approved by the Institutional Animal Care and Use Committee of HUST [IACUC_Number:S2344].

### 2.11 Micronucleus assay

The micronucleus (MN) assay in ESCC cells was conducted using the In Vitro MicroFlow Kit (Litron, USA), which is a two-color labeling technique. Briefly, KYSE30 and KSYE150 cells at a density of 2.5 × 10^4 per well were seeded into six-well cell plates. After 24 h transfection, Nucleic Acid Dye A (EMA) and Nucleic Acid Dye B (SYTOX Green) were added to stain compromised outer membrane and chromatin of ESCC cells, respectively. Subsequently, we applied Complete Lysis solution to digest these stained cells. Finally, we used flow cytometric (LSRII, BD Biosciences, USA) to analyze the proportion of two-color labeling cells and calculated the differential staining between healthy chromatin and dead/dying cells.

Additionally, we also applied another method cytokinesis block to evaluate the MN proportion in ESCC cells or healthy individuals. For ESCC cells, cell suspension was harvested after 24 h transfection. For healthy individuals, each subject provided 0.5 ml of blood, which was supplemented with heparin and 4.5 ml of culture medium containing 15% fetal calf serum, penicillin (100 IU/ml) and phytohemagglutinin (20 μg/ml; Sigma, USA). Furthermore, the cell cultures were added into a cytochalasin B (6 μg/ml; Sigma, USA) and incubated for 3 days. Then, we prepared cell slides and stained cells with 10% Giemsa solution. Finally, we counted the proportion of micronucleated binucleated cells in 1000 binucleated lymphocytes, as well as micronucleated ESCC cells in 1000 tumor cells under a light-microscopy.

### 2.12 The assays for testing the effect of MCM10 inhibitors on KYSE30 and KYSE150 cells

To assess the efficacy of several MCM10 inhibitors Suramin and its analogues (NF157, NF546 and PPADS) on ESCC cells proliferation and migration abilities, we firstly determine the half-maximal inhibitory concentration (IC_{50}) of these inhibitors by measuring the cell viability at different time points. Drug treatment was started 24 h post-seeding and continued for 48 h, and then ESCC cells were harvested and used for testing the cell viability. Furthermore, Suramin (Cat# HY-B0879, MedChemExpress, USA), NF157, NF546, and PPADS (Cat# 2450, Cat# 3892 and Cat# 0625, R&D Systems, USA) at a concentration of IC_{50}, respectively, were added into ESCC KYSE30 and KYSE150 cells for incubation 48 h, and then cells were harvested and used for cell proliferation and migration testing.

### 2.13 Statistical analysis

The Cox regression analysis under an additive genetic model was conducted to calculate the associations of candidate germline variants with survival status of ESCC subjects with adjustment for some confounders, such as age, sex, smoking status, drinking status, and tumor stage. The comparison of overall survival of ESCC between groups with low MCM10 expression and high MCM10 expression was performed by Kaplan–Meier survival analysis and the corresponding p values, hazard ratios (HRs), and 95% confidence intervals (CIs) were determined by the log-rank test. Besides, we used the paired Student’s t test to compare the differences between both matched groups, and applied the Student’s t-test to test the differences between both non-matched groups, respectively. The one-way variance analysis was applied to compare the homogeneity of variance among the groups. For functional experiments, each experiment was performed independently three times, each with triplicates at least. All statistical calculation was conducted by R (3.3.0) or SPSS software (21.0).

### 3 RESULTS

#### 3.1 MCM10 rs2274110-AA genotypes confer an inferior survival of ESCC patients

To identify promising prognostic biomarkers, we firstly carried out an exome-wide association analysis with survival of 904 ESCC patients to identify candidate SNPs-correlated with ESCC prognosis (Figure S1). The quantile-quantile plot showed that there is a good match between the distributions of the observed p values and the expected ones by chance; and a small genomic control factor revealed a minimal inflation of genome-wide association significance (λ = 1.052; Figure S1A). Strikingly, a missense variant rs2274110 located at the 15th exon of MCM10 was identified to confer a poor survival of ESCC patients with
FIGURE 1  MCM10 rs2274110[A] is associated with the poor prognosis of ESCC. (A-C) Kaplan-Meier estimates of survival time for patients with ESCC stratified by MCM10-rs2274110 genotype in discovery stage (A), replication stage (B), and combined stage (C) consisting of the discovery and replication stage. (D) Kaplan-Meier estimates of survival time for patients with ESCC from TCGA dataset stratified by MCM10-rs2274110 genotype. $p$ values, HRs, and 95% CIs were calculated using Cox regression analysis with adjustments for age, sex, smoking status, drinking status, and tumor stage in (A-C), and using log-rank test in (D).

A promising association (Figure S1B). ESCC subjects carrying rs2274110-AA genotypes tended to confer an inferior overall survival, compared to those with rs2274110-AG/GG genotypes ($p = 4.39 \times 10^{-6}$, HR = 1.65, 95% CI = 1.33–2.05, Figure 1A). The association is successfully replicated in an independent cohort with 503 ESCC patients with $p$ values being $2.91 \times 10^{-3}$ (HR = 1.65, 95% CI = 1.19–2.30, Figure 1B). In addition, we further combined the results from both stages and still found that compared with individuals carrying the rs2274110-AG/GG genotype, the patients with AA genotype present a shorter survival time of ESCC with HR being 1.61 (95% CI = 1.35–1.93, $p = 1.35 \times 10^{-7}$, Figure 1C). Consistently, these results are further validated in ESCC patients obtained from The Cancer Genome Atlas (TCGA) database (HR = 2.78, 95% CI = 1.29–6.00, $p = 0.038$, Figure 1D). Moreover, we also evaluated the association of rs2274110 genotypes with tumor stage of ESCC patients and interestingly found that rs2274110-AA genotype carriers have a more advanced tumor stage, compared to the subjects with rs2274110-AG/GG genotypes (Table S3). Taken together, these results provide population evidence supporting that ESCC patients carrying rs2274110-AA genotype had an inferior prognosis and might harbor an aggressive tumor subtype.

3.2  MCM10-rs2274110[A] variant stabilizes MCM10 protein expression by increasing its SUMOylation level

Considering that variant rs2274110 is located at the 15th exon of MCM10, we speculated that it might have a regulatory effect on gene expression or post-translational modifications (PTMs) of protein. When we overexpressed the same amount of transcript with MCM10-rs2274110[A] allele or MCM10-rs2274110[G] allele in KYSE30 and KYSE150 cell lines carrying rs2274110-AA genotype, respectively, there are no significant differences in mRNA level of MCM10 between both transcripts (Figure 2A). However, the protein level of MCM10 is higher in group with MCM10-rs2274110[A] allele overexpression, compared with the group with MCM10-rs2274110[G] allele overexpression (Figure 2B). In addition, we also measured the expression of MCM10 protein in ESCC samples...
FIGURE 2 MCM10-rs2274110[A] variant stables the expression of MCM10 protein by increasing its SUMOylation level. (A and B) The mRNA (A) and protein expression (B) levels of MCM10 were detected by qRT-PCR and western-blotting assays, respectively, in KYSE30 and KYSE150.
with different rs2274110 genotypes and observed that the rs2274110-AA genotype carriers have a higher MCM10 protein expression, compared to the rs2274110-AG genotype carriers (Figure S2D). Interestingly, we further found that this variant in the MCM10 can cause a Lys[K]669Arg[R] change and disrupt a SUMOylation site at K669 of MCM10 protein (Figure 2C). Through a mass spectrometry assay followed by overexpression of transcripts with different MCM10-rs2274110 alleles, we further observed that the SUMOylation level of MCM10 is higher when MCM10-[A] allele overexpression, compared with that of MCM10[G] allele overexpression (Figure 2D). Additionally, the Co-IP assay also showed that the binding of SUMO2/3 is more enriched with the MCM10[A], compared with the MCM10[G] (Figure 2E). Interestingly, when knocking down SUMO2/3 with siRNAs, the differences of MCM10 protein levels between MCM10[A] allele and MCM10[G] allele are substantially attenuated in KYSE30 and KYSE150 cells (Figure 2F). Furthermore, we observed that the degradation rate of MCM10 protein is also shown to be lower for the MCM10[A] group, compared with the MCM10[G] group, in both ESCC cells (Figures 2G and 2H). Collectively, these results indicate that the MCM10-rs2274110[A] allele can increase its SUMOylation level to inhibit the degradation of MCM10 protein.

3.3 | The high expression of MCM10 confers an inferior survival of ESCC patients

We further evaluated whether the overexpression of MCM10 is also associated with the clinical prognosis of ESCC patients. In contrast to adjacent normal tissues, the expression of MCM10 was shown to be increased in tumor tissues of ESCC from four independent cohorts, including TCGA/GTEX, GEO, and our own ESCC samples, respectively (Figures 3A-3D). Moreover, MCM10 expression was presented extensively to be upregulated level in tumor tissues of other cancer types, and the amplification of MCM10 also frequently occurred across multiple cancer types, including ESCC, from the TCGA database (Figures S3A and S3B). Also, we found that MCM10 presented a high dependence degree in two different esophagus cancer cell line TE4 and TE6 based on the loss-of-function screens data of CRISPR/Cas9 technology (Figure 3E), suggesting the MCM10 is essential for ESCC cells proliferation. Furthermore, the high expression of MCM10 is remarkably associated with multiple clinical prognosis traits of ESCC, such as the lymph node (L.N.) metastasis, advanced tumor stage, and poor survival (Figures 3F-3I). In addition, in consistent with results in ESCC, the significant associations between high expression of MCM10 and the poor survival outcomes were observed across multiple cancer types (Figure S4). Collectively, these results demonstrate that the MCM10 may not only be a prognostic marker but also play an important role in promoting ESCC progression.

3.4 | Upregulation of MCM10 deteriorates the progression and metastasis of ESCC cells

To further illuminate the functional role of MCM10 in the progression of ESCC, we tested the ESCC cell malignant phenotypes upon the knockdown, knockout of MCM10, or overexpression of transcripts carrying different MCM10-rs2274110 allele, respectively. We observed that the overexpression of MCM10[A] allele can prominently enhance the proliferation ability of both ESCC cells, in contrast to the overexpression of MCM10[G] allele or the control vector (Figure 4A). While both MCM10 knockdown by siRNAs and MCM10 knockout by CRISPR/Cas9 significantly inhibited the proliferation rate of both ESCC cells (Figure 4A). Consistently, the results present similar trends in the colony formation assays (Figure 4B). Similarly, in the trans-well assays, the ESCC cells also showed higher migration ability in group with overexpression of MCM10[A] allele, compared to the MCM10[G] allele or the control vector group (Figure 4C). Expectedly, we also...
FIGURE 3 The association of MCM10 with clinical prognosis of ESCC patients. (A-D) The expression of MCM10 is significantly upregulated in ESCC tumor tissues compared with their normal tissues from multiple cohorts including TCGA/GTEX (A), Hu (GSE20347) (B), Su (GSE23400) (C), and our own ESCC tissues (D). Data were shown as the mean ± SD, and all **p < 0.01 values were calculated by a two-sided Student’s t-test in TCGA/GTEX ESCC tissues, but with a paired two-sided Student’s t-test in Hu (GSE20347), Su (GSE23400), and our own ESCC tissues. (E) MCM10 is essential for cell growth with higher CERES scores in ESCA TE4 and TE6 cell lines from the data of genome-wide CRISPR/Cas9-based loss-of-function screen. Higher CERES scores demonstrate an elevated dependency of cell viability on given genes. (F and G) MCM10 expression levels were measured in ESCCs with or without lymph node (L.N.) metastasis (F) or different tumor stages (G) of ESCC from TCGA database. Data were presented as the mean ± SD, and p values were calculated by using a two-sided Student’s t-test. (H and I) Kaplan-Meier estimates of survival time for individuals with ESCC stratified by MCM10 expression from Peter esophagus (GSE19417), and ICGC database. p value and HR (95% CI) were calculated by the log-rank test. Abbreviation: ICGC, International Cancer Genome Consortium cohort.
FIGURE 4  Upregulation of MCM10 promotes the proliferation and metastasis of ESCC cells. (A) The effect of MCM10[A] and MCM10[G] overexpression, MCM10 knockdown or MCM10 knockout on the proliferation rate of KYSE30 and KYSE150 cells. Data were
found that the knockdown or knockout of MCM10 can significantly attenuate the progression ability of ESCC cells (Figures 4D and 4E). Furthermore, we also found that the MCM10 promotes ESCC metastasis in vivo. The lung metastasis model revealed that the stable overexpression of MCM10 in both KYSE30 and KYSE150 cells can significantly facilitate colonization of the lung and increases the number of lung metastases, whereas the knockdown of MCM10 in both ESCC cells substantially inhibited these effects (Figures 4F and 4G). These findings in vitro and in vivo collectively reveal that the upregulation of MCM10 substantially enhances ESCC cells growth and migration.

3.5 | Upregulation of MCM10 induces the genomic instability of ESCC cells

Previous researches showed that the deregulation of CDT1 or depletion of its inhibitor geminin may lead to the DNA over-replication and caused genomic instability.17,32,33 As MCM10 has synergistic effect with CDT1 in regulating replication licensing, we then tested whether the MCM10 enhances ESCC progression by inducing the genomic instability. We interestingly observed that the proportion of micronucleated KYSE30 and KYSE150 cells are significantly increased in the group with the overexpression of MCM10[A] allele, compared with the group with over-expression of the MCM10[G] allele or the control vector (Figures 5A and 5B). When MCM10 was successfully knocked down, we found a significantly decreasing in the proportion of both the micronucleated ESCC KYSE30 and KYSE150 cells (Figures 5C and 5D). Expectedly, these results are consistent with the results measured with another micronucleus testing method (Figures 5E-5G). In addition, we further measured the proportion of micronucleated lymphocytes in 105 healthy subjects carrying different rs2274110 genotypes and found that subjects with AA genotype have a more amount of micronucleated lymphocytes, compared the subjects carrying AG/GG genotypes (Figure 5H). Collectively, these results demonstrate that aberrant upregulation of MCM10 may facilitate the progression of ESCC through inducing the genomic instability.

3.6 | The MCM10 inhibitors Suramin and its analogues may serve as potential anti-cancer agents for ESCC

Furthermore, we aimed to evaluate whether previously reported MCM10 inhibitors Suramin and its analogues (NF157, NF546, and PPADS)29,30 could inhibit ESCC cells growth and metastasis. The results revealed that these molecule inhibitors treatments can effectively inhibit the cell viability of both KYSE30 and KYSE150 cells, and presented in a dose dependent manner (Figure 6A). Moreover, the cell proliferation and migration abilities of both KYSE30 and KYSE150 cells were significantly suppressed under these drugs treatment (Figures 6B and 6C). Interestingly, in these several drugs, the NF157 had the most effective and resulted in a 69.03% or 66.41% decrease in the migration ability of KYSE30 or KYSE150 cells, respectively. Collectively, these findings indicate that the MCM10 inhibitors Suramin and its analogues can effectively suppress the oncogenic transformation induced by ESCC cells.

4 | DISCUSSION

Through comprehensively integrating two-stage population survival analysis and multipronged biochemical experiments in this study, we identified a germline variant rs2274110 in MCM10 that confers an inferior survival of ESCC patients. This functional variant can upregulate SUMOylation level of MCM10 protein to result in its protein aberrant overexpression, which further substantially facilitate ESCC progression and metastasis in vitro and in vivo probably by fueling DNA over-replication and genomic instability. Importantly, MCM10 is shown to be served as a promising prognostic biomarker and MCM10 inhibitors Suramin, and its analogues are revealed to present potential anti-cancer effects for ESCC.

Reversible PTMs of protein have been well documented as important biological mechanisms that dynamically and rapidly response to extracellular and intracellular stimulation and influence evolutionary development and progression of diseases.34 SUMOylation is one of PTMs and revealed to be closely involved in various physiological
FIGURE 5  Upregulation of MCM10 induces the genomic instability of ESCC cells. (A-D) The effect of MCM10[A] and MCM10[G] overexpression (A and B) or MCM10 knockdown (C and D) on MN frequency in KYSE30 and KYSE150 cells by flow cytometric analysis. Flow cytometry images (A and C) and quantitative statistics (B and D) were shown as the means ± SD from a representative result of three repeated experiments and each with six replicates. **p < 0.01 was calculated by a two-sided Student’s t-test. (E-G) The effect of MCM10[A] and MCM10[G] overexpression (F) or MCM10 knockdown (G) on MN frequency in KYSE30 and KYSE150 cells by cytokinesis block (CB) micronucleus testing. Representative images (E) and quantitative statistics (F and G) were shown as the as the medians (min to max) from three repeated experiments, each with triplicates. **p < 0.01 was calculated by a two-sided Student’s t-test. (H) Carries with the MCM10-rs2274110 AA genotype had higher MN frequency in 105 healthy individuals. Results were shown as the medians (min to max), and p value was calculated using a two-sided Student’s t-test.
FIGURE 6  The MCM10 inhibitors Suramin and its analogues inhibit cell proliferation and migration of ESCC cells. (A) Dose–response curves of KYSE30 and KYSE150 cell lines treated with Suramin and its analogues (NF157, NF546, and PPADS) with an endpoint measurement at 48 h. Data were shown as the means ± SD from three repeated experiments, each with triplicates. IC50, half-maximal inhibitory concentration. (B) The effect of the Suramin and its analogues on the proliferation rate of ESCC cell lines. Results were shown as the mean ± s.e.m. from three experiments, each with six replicates. *p < 0.05, **p < 0.01, compared with controls by a two-sided Student’s t-test. (C) The effect of Suramin and its analogues on cell migration ability of ESCC cells. Results were shown as the mean ± SD from three repeated experiments, each with triplicates. **p < 0.01 was calculated using a two-sided Student’s t-test.
processes of mammalian cells, such as signal transduction, genome stability, the DNA damage responses and protein trafficking, and one that seems to be upregulated in many diseases. Interestingly, in contrast to ubiquitylation of protein, SUMOylation does not degrade target proteins, while seems to modulate their subcellular compartmentalization and reinforce their stability. Additionally, Bao et al also revealed that the K501 SUMOylation on BACE1 protein enhances its stability and escalates the protease activity in Alzheimer’s disease. In consistent with these findings, we also found that the rs2274110[A] variant stables MCM10 protein expression by increasing its SUMOylation level and presents a shorter survival outcome for ESCC. These findings underline the clinical potential and importance of targeting the SUMOylation machinery for disease therapy.

Replication licensing is a well-organized process to assure the proper duplication and assembly of genome during cell cycle progression. Recently, it was suggested that both the reduction and the ectopic or increasing of origin licensing could result in replication stress and re-replicated process of DNA, thus resulting in occurrence of genomic instability. Especially, in cancer cells, most of the replication licensing factors were upregulated, and the DNA re-replication (over-replication) was induced. The DNA re-replication leads to the accumulation of single-strand DNA (ssDNA) and the formation of DNA double-strand breaks (DSBs), which are sources of genomic instability.

The MCM family genes are essential factors in eukaryotic genome replication initiation and elongation by activating and stabling the C45-MCM2-7-GINS (CMG) complex and leading to DNA unwinding and replication fork assembly. It was reported that in normal cells of eukaryotes, loss or mutations in the MCMs are associated with genomic instability, possibly due to a failure to license additional replication origins following a replication fork failure. However, in tumor cells, depletion of the MCMs, for example MCM7, was found to suppress the tumor growth and progression of non-small cell lung cancer and glioblastoma in vitro and in vivo. As for MCM10, it frequently presents overexpression and amplification in multiple cancer types and contributes to poor prognosis. Moreover, the MCM10 expression is activated by some oncogenes, such as N-MYC and Ewing’s sarcoma-derived oncogenes in neuroblastoma and Ewing’s tumors. Intriguingly, MCM10 overexpression in yeast had also been reported to drive genome instability. In consistent with these finding, here our study also found that MCM10 is overexpressed in tumor tissues of ESCC and prominently correlated with the inferior survival of ESCC patients. The aberrant overexpression of MCM10 was shown to significantly provoke ESCC cells proliferation and migration abilities and fuel more micronucleated ESCC cells. These observations suggested at least two hypotheses for the function of MCM10 in ESCC: First, the upregulation of MCM10 may simply be one of replication licensing factors in response to the rapid increasing rate of proliferation in tumor cells which need MCM10 to overcome limitations for DNA replication dictated by active cell replication and altered cell cycle control. Second, aberrant overexpression of MCM10 might act as an augmenting force in cancer development or progression to drive over-replication and genomic instability. Considering that aberrant overexpression of MCM10 in ESCC tumor cells might induce DNA over-replication, moderately decreasing expression of MCM10 might be helpful for suppressing the proliferation and migration of ESCC cells. Noticeably, whether MCM10 plays a causal role in cancer cells proliferation, migration or genome instability is an interesting issue, which should be further interrogated.

MCM10, as a replication licensing factor, appears to be a promising anticancer drug target. Previous researches support this idea revealing that in contrast to normal cells, the ablation of replication licensing factors can effectively reduce replication stress specifically in cancer cells. In addition, a high-throughput cell-based screening for licensing inhibitors also prioritized a small-molecule inhibitor for replication licensing factor, RL5a, which can restrain the binding of ORC to DNA and compromise MCM2–7 loading onto origins, thereby successfully reducing the proliferation of cancer cells. Moreover, licensing inhibitors might also be shown to work synergistically with existing chemotherapeutic drugs. These findings pave the way for replication licensing factors serving as an attractive anti-cancer target. In our study, we also indicated that the MCM10 inhibitors Suramin and its analogues (NF157, NF546, and PPADS) can block both cancer cell proliferation and migration in the ESCC. Besides, previous researches have also revealed that Suramin can effectively inhibit growth of tumor xenografts in nude mice, such as osteosarcoma, breast cancer, and Ewing’s sarcoma and moderately extend the lifespan of animals with breast cancer lung metastasis. Additionally, suramin has also been demonstrated to enhance paclitaxel activity against tumor growth or metastasis of both lung cancer and breast cancer in vivo. These findings provide evidence suggesting that MCM10 small-molecule inhibitors may serve as potential anti-cancer agents in treating advanced cancer. However, it should be noted that Suramin and its analogues exert a wide variety of biological effects and that are not specific to MCM10 alone. They have also been found to inhibit other proteins involved in other tumor signaling pathways, such as DNA polymerases, Wnt, and insulin-like growth factor-I receptor. A phase I/II clinical trial related to suramin therapy in hormone refractory prostate cancer patients also uncovered that...
suramin had a neurologic toxicity, although antitumor activity was observed. Further investigations are warranted to deconstruct the precise mechanisms and effective targets of this drug before its clinical use. In addition, considering that MCM10 inhibitors Suramin and its analogues (NF157, NF546, and PPADS) are not specific to MCM10 alone, researchers in many disciplines are encouraged to find and deconstruct new specific drugs in the future.

In summary, through a two-stage survival analysis for 1407 ESCC patients, we identified a replication licensing factor germline variant, which might be ideal as a potential prognostic biomarker stratifying ESCC patients effectively. Mechanistically, we further demonstrated that replication licensing factor MCM10 can deteriorate the progression and metastasis of ESCC and induce DNA over-replication and genomic instability. These findings collectively indicate that preexisting hereditary genetics of individuals can effectively influence progression and prognosis outcomes of ESCC and provide more sights into the personalized therapy for cancer.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Informed consent was obtained from all participants, and this study was approved by the institutional review board of HUST ([2016]IEC(S160)). All animal experimental procedures were performed in accordance with the relevant institutional and national guidelines and approved by the Institutional Animal Care and Use Committee of HUST [IACUC_Number: S2344].

CONSENT FOR PUBLICATION
Consent to publish has been obtained from all authors.

DATA AVAILABILITY STATEMENT
The MCM10 mRNA expression data were derived from the TCGA Research Network (http://cancergenome.nih.gov/), GTEx (https://commonfund.nih.gov/GTEx/), and GEO database (https://www.ncbi.nlm.nih.gov/geo/). Other data associated with this study are present in the paper and Supplementary information or are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST
The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS
Xiaoping Miao and Jiang Chang were the overall principal investigators in this study who conceived the study and obtained financial support, were responsible for the study design and supervised the entire study. Jianbo Tian and Jiang Chang performed statistical analyses, interpreted the data, and drafted the initial manuscript. Jianbo Tian, Zequn Lu, Siyuan Niu, Shanshan Zhang, Pingting Ying, Lu Wang, Ming Zhang, Yimin Cai, Tianyi Dong, and Ying Zhu performed laboratory experiments. Rong Zhong, Zhihua Wang, and Xiaoping Miao were responsible for patient recruitment and sample preparation. All authors approved the final report for publication.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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