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Pulrodemstat, a selective inhibitor of KDM1A, suppresses head and neck squamous cell carcinoma growth by triggering apoptosis

Cheng Jiang[1†], Xiaofeng Weng[2†], Yuqing Chen[3*] and Junjun Yang[4*]

Abstract

Background Chemotherapy is often ineffective as a first-line treatment for head and neck squamous cell carcinoma (HNSCC), and a more precise and effective therapeutic option is urgently needed.

Methods High-throughput screening of a histone demethylase inhibitor library was performed to identify potential drugs for treating HNSCC. The Cancer Genome Atlas (TCGA) and single-cell sequencing were used to evaluate the potential diagnostic value and expression distribution of candidate drug targets. Colony formation, transwell assays, and flow cytometry analyses were used to assess the antitumor function of the potential drugs. The CCK-8 assay was used to compare the antitumor activity of the candidate drug and the traditional chemotherapy drug. Bioinformatic analysis based on TCGA database was used for unveiling the upstream signaling.

Results Pulrodemstat, a selective KDM1A inhibitor that is ongoing clinical trial, stood out as the most effective candidate anti-HNSCC drug based on the high-throughput screening. IC_{50} analysis revealed that Pulrodemstat might possess stronger anti-tumor activity than 5-Fu. Additionally, Pulrodemstat dramatically suppressed HNSCC cell proliferation and migration without inducing toxicity in normal cells. TCGA analysis revealed that KDM1A is positively associated with tumor proliferation, DNA repair, and DNA replication in HNSCC. Consistent with these results, Pulrodemstat substantially induced apoptosis in the HNSCC cells. Furthermore, TCGA analysis revealed that KDM1A was aberrantly overexpressed in HNSCC, positively correlated with malignancy, and negatively associated with the clinical outcomes of HNSCC patients. Notably, single-cell analysis indicated that KDM1A was mainly distributed in the malignant cells of HNSCC samples, highlighting that Pulrodemstat may be a more precise therapeutic option for HNSCC. In addition, methylation occupancies in the KDM1A promoter were substantially low in HNCC tumors, and low methylation occupancies in the KDM1A promoter predicted poor clinical outcomes in HNSCC. These data are consistent with the KDM1A expression in HNSCC. Moreover, TET3, a DNA demethylase, was strongly and positively correlated with KDM1A expression.

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Conclusions Pulrodemstat is an effective therapeutic drug for HNSCC. Thus, the TET3/KDM1A axis may account for the malignant phenotype of HNSCC.

Keywords Head and neck squamous cell carcinomas, Pulrodemstat, Lysine Specific Demethylase 1, DNA demethylation, Tet Methylcytosine Dioxygenase 3

Background

Head and neck squamous cell carcinoma (HNSCC) is one of the most malignant cancers and contributes to an increasing number of deaths [1]. The traditional first-line treatment for HNSCC is chemotherapy or radiotherapy (RT). Although chemotherapy prolongs median overall survival, its non-selective killing function and drug resistance limit its therapeutic efficacy [2]. Owing to the increasing number of HNSCC patients, there is an urgent need to explore novel, more precise drugs that will optimize current treatments and prolong the survival rates of HNSCC patients.

Epigenetic regulation, including post-translational histone, DNA, RNA, and noncoding RNA modifications, is essential for diverse biological processes [3]. Epigenetic dysregulation is a hallmark of cancer [4]. Accumulating evidence suggests that histone methylation erasers are attractive therapeutic targets for various cancer types [5]. Among histone modifier, KDM1A had been reported to facilitate tumor progression across human cancer types [6]. KDM1A inhibitors exhibit strong antitumor functions in both hematologic and solid tumors, and some KDM1A inhibitors have been used in clinical trials [7]. In this regard, a high-throughput histone demethylase inhibitor library screening was conducted to identify the novel anti-HNSCC drugs.

DNA demethylation facilitates oncogenes transcription [8]. All DNA demethylases, including TET1, TET2, and TET3, had been indicated to promote carcinogenesis by removing silencing methylation markers distributed in the CpG islands of a series of oncogenes [8]. Moreover, crosstalk between DNA demethylation and histone demethylation has been established to promote tumor development [9].

Here, we identified a novel anti-HNSCC drug, Pulrodemstat, using high-throughput screening for histone demethylase inhibitors. Functional analysis revealed that Pulrodemstat possessed a more powerful antitumor function than traditional chemotherapy drugs by assessing colony formation and migration capacity. In addition, KDM1A as the selective target of Pulrodemstat, was found to be highly expressed in tumor tissues and positively associated with poor clinical outcomes of HNSCC patients. Notably, our clinical relevance analysis revealed that TET3 might be the culprit accounting for the aberrantly increased KDM1A transcripts by removing the silenced DNA methylation markers of KDM1A CpG islands.

Materials and methods

Cell culture

Cal-27 and SCC-9 were obtained from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS (Gemini). SCC-1 cells (Cat No.: CL-0907) were purchased from Procell (Wuhan, China) and cultured in RPMI-1640 (Gibco) containing 10% fetal bovine serum (FBS). FaDu cells (Cat No.: CL-0083) were obtained from Procell and cultured in NEAA (Cat No.: PB180424; Procell)-containing MEM (Gibco) supplemented with 10% FBS. Het1A cells were gifted by Shuang Han (Affiliated Hospital of Jiangnan University). Cells were maintained at 37 °C in a saturated humid atmosphere containing 95% air and 5% CO_2.

Lentivirus-mediated gene silencing

HEK293T cells were co-transfected with the shRNA in order to produce the lentivirus. Following a 48-h transfection period, the viral supernatant was collected, filtered through a 0.45 μm filter, and subsequently used to infect target cells that had reached 80% confluence, with protamine sulfate (8 μg mL^{-1}) present. The KDM1A shRNAs were kind gifts from Dr. Zhicheng Gong (Affiliated Hospital of Jiangnan University, Jiangsu).

CCK-8 assay

After treatment, the medium was replaced with 100 μL fresh medium containing 10 μL CCK-8 reagent and then incubated in 37 °C for 1 h. After incubation, cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

Drug screening

Histone demethylase inhibitor library was purchased from MedChemExpress (Shanghai, China), which was pre-dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stocked at −80 °C according to the product data sheets.

Cal-27, SCC-9, and Het1A cells were seeded in a 96-well plate at 3000 cells per well and cultured for 24 h. After 24 h, the media was replaced with the drug-containing media comprising DMEM and 10 μM of each inhibitor from the library and then cultured for another 96 h. Cell viability was determined using a CCK-8 assay and visualized using a heatmap.

IC_{50} calculation

Cal-27, SCC-9, Het1a, SCC-1 and FaDu cells were seeded in a 96-well plate at 3000 cells per well. Next day, the medium containing a gradient dose of Pulrodemstat (up to 5 μM) was subjected to use for further culture in the subsequent four days. After culturing for the indicated times, the inhibition rates were calculated based on cell viability determined using the CCK-8 assay.

5-Fu IC_{50}

The IC_{50} of 5-Fu in HNSCC cell lines were obtained from the Genomics of Drug Sensitivity in Cancer database (https://www.cancerrxgene.org/).

Colony formation assay

Cal-27 and SCC-9 cells were plated in a 6-well plate at 2000 cells per well. After incubation for 24 h, the cells were treated with the indicated IC_{50} of Pulrodemstat for 1 week, and the medium containing Pulrodemstat was added every 2 days. After culturing for the indicated time periods, the cells were fixed with methanol for 10 min and then stained with crystal violet.

Transwell assay

An 8 μm pore Boyden chamber (Corning Costar, 3422) was used to assess in vitro migration capacity. Cells (200 μL, 1×10^5) were plated in serum-free DMEM in the upper chamber, and 500 μL of 10% FBS was added to DMEM as a chemoattractant in the lower chamber. Cells on the upper side were carefully discarded, and those adhering to the underside of the membranes were fixed in methanol and stained with crystal violet. The number of migrated cells was counted under a microscope and five contiguous fields of each sample were examined using a 20 × objective.

Flow cytometry-based apoptosis analysis

Cells after being treated with DMSO or Pulrodemstat were collected and then resuspended by 100 μL of FITC-Annexin V and PI solution (MedChemExpress). Finally, all cell samples were analyzed using an ACEA NovoCyte (Agilent) and the data was analyzed by NovoExpress.

TCGA analysis

TCGA-based expression and correlation analyses were performed using Gene Set Cancer Analysis (http://bioinfo.life.hust.edu.cn/GSCA/#/expression).

Single cell analysis

Single-cell analyses (GSE150430 and GSE148673) were performed using the Tumor Immune Single-cell Hub 2 (TISCH2, http://tisch.comp-genomics.org/home/).

Statistical analysis

Data were obtained from at least three independent experiments. Student's *t*-test was used to determine the significance between groups. $P<0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8 software.

Results

Histone demethylase inhibitor screening indicates Pulrodemstat as a potent anti-HNSCC drug

To explore whether histone demethylase inhibitors could serve as potent anti-HNSCC drugs, we screened histone demethylase inhibitors in the HNSCC cell lines, Cal-27 and SCC-9, as well as in human esophageal normal cells, Het1A. As represented in Fig. 1A, thirty-six preclinical histone methylation "eraser" inhibitors were subjected to screening and the anti-tumor capacity was evaluated by cell viability. Based on this screening, the results were visualized using a heatmap (Fig. 1B). Interestingly, 7 of the 36 exhibited strong antitumor capacity (Fig. 1B). Notably, Corin, a dual inhibitor of HDAC1/KDM1A,

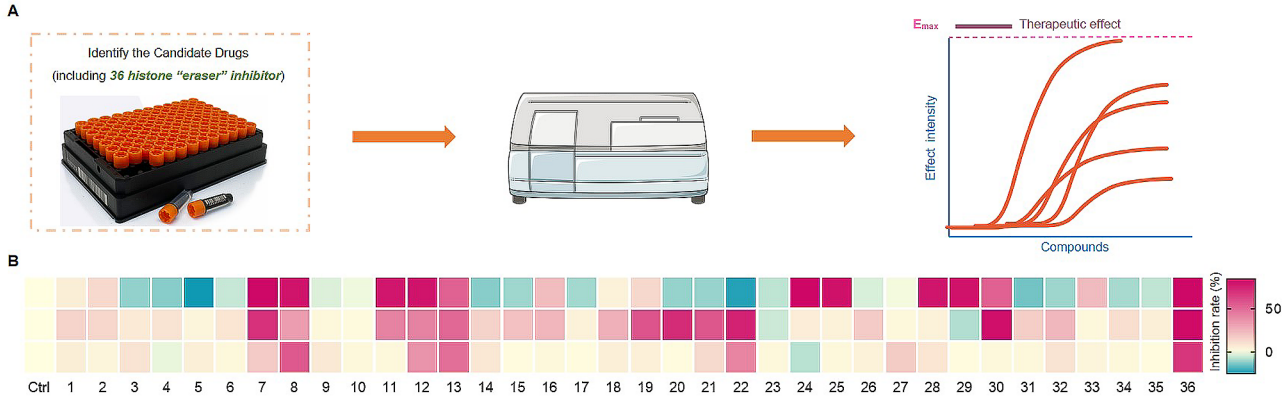

Fig. 1 Pulrodemstat is a potent anti-HNSCC drug. (**A**) The workflow of histone demethylase inhibitor screening. (**B**) The inhibition rates of each inhibitor in Cal-27, SCC-9 and Het1A cells were visualized by the Heatmap. The color intensity positively correlated with the inhibition rates

Pulrodemstat, a KDM1A-specific inhibitor, and GSK-J4, a JMJD3 inhibitor, remarkably suppressed HNSCC cell viability without affecting normal cells (Fig. 1B). Collectively, our study identified a novel and selective anti-HNSCC drug from an epigenetic perspective.

Pulrodemstat suppresses HNSCC progression

Pulrodemstat, a KDM1A inhibitor, is in an ongoing Phase II clinical trial for both hematological and solid tumors [10]. Hence, Pulrodemstat was selected for further studies. We determined the IC_{50} of Pulrodemstat in HNSCC cell lines, Cal-27 and SCC-9, and found the IC_{50} of Pulrodemstat was only 2.42 and 0.52 μM which was much lower than the median IC_{50} of 5-Fu, the first-line treatment for HNSCC (Fig. 2A–D). Furthermore, the anti-proliferative role of Pulrodemstat was confirmed in additional HNSCC cell lines, including SCC-1 and FaDu (Fig. S1A). Notably, the IC_{50} of Pulrodemstat in Het1A cells was much higher than that in HNSCC cells (Fig. S1B). Moreover, the clonogenic assay revealed that Pulrodemstat selectively suppressed HNSCC cell growth without affecting the growth of normal cells, as evidenced by the drastically decreased colony formation capacity of HNSCC cells, but not Het1A cells, under the same dose of Pulrodemstat (Fig. 2E). Meanwhile, depletion of KDM1A drastically suppressed the clone formation capacity of HNSCC cells (Fig. 2F, G). KDM1A-depleted cells were then treated with Pulrodemstat (Fig. 2H). Notably, Pulrodemstat failed to further suppress the growth of KDM1A-silenced HNSCC cells, highlighting that KDM1A is the core target for Pulrodemstat to exert its anti-HNSCC effects (Fig. 2I). These findings suggest that Pulrodemstat may serve as a novel and selective therapeutic option for HNSCC.

HNSCC has a high invasive propensity and proclivity for metastasis to the cervical lymph nodes, which ultimately results in relapse and mortality [11, 12]. Aberrant upregulation of KDM1A promotes cancer metastasis [13–15]. Based on these findings, we hypothesized that Pulrodemstat treatment suppresses the migration capacity of HNSCC cells. Fortunately, we found that the number of migrated cells remarkably decreased upon Pulrodemstat treatment, as evidenced by the reduced number of crystal violet-positive cells (Fig. 2J, $p<0.001$). Collectively, our findings suggest that the KDM1A inhibitor Pulrodemstat may be a novel selective therapeutic option for patients with HNSCC.

Pulrodemstat induces apoptosis by disrupting the DNA replication process

To further explore the molecular mechanism underlying the inhibition of anti-HNSCC activity by KDM1A repression, we performed a series of bioinformatics analyses. Consistent with the observed phenotype, KDM1A expression was significantly and positively correlated with proliferation-associated gene sets in HNSCC, highlighting the pivotal role of KDM1A in maintaining the rapid growth of HNSCC (Fig. 3A). Furthermore, we found that HNSCC cells with high KDM1A expression might possess a strong DNA repair capacity and fast DNA replication frequencies, which supports the rapid proliferation of HNSCC cells, as indicated by the association between KDM1A expression and DNA repair and replication (Fig. 3B, C). Impaired DNA repair and replication drives apoptosis [16]. Hence, we hypothesized that Pulrodemstat treatment induces apoptosis in HNSCC cell lines. As expected, Pulrodemstat treatment led to a significant induction of apoptosis, as evidenced by a drastic increase in the number of PI-positive cells (Fig. 3D). Collectively, our data suggest that Pulrodemstat might blunt DNA replication and repair to drive apoptosis and repress HNSCC progression.

KDM1A serves as a potential predictive biomarker for HNSCC

To assess the clinical relevance of KDM1A in HNSCC, we employed TCGA and Single cell RNA-sequencing analyses to achieve our aim. Consistent with previous reports that KDM1A is aberrantly upregulated in human cancers [13–15, 17], we found that *KDM1A* transcript levels were dramatically higher in HNSCC tumor tissues than in normal tissues (Fig. 4A). In addition, our data revealed that KDM1A expression positively correlated with HNSCC malignancy (Fig. 4B). Given that KDM1A expression levels are negatively correlated with clinical outcomes in several human cancer types (ref), we analyzed the correlation between KDM1A expression and the clinical outcomes of HNSCC and found that aberrantly elevated KDM1A expression was associated with poor clinical outcomes (Fig. 4C). Several KDM1A inhibitors have been validated as highly selective therapeutic options for human cancers (ref); however, the underlying mechanism is largely unknown, especially for HNSCC. Therefore, we analyzed the KDM1A expression distribution in HNSCC tumor tissues using single-cell RNA sequencing. Interestingly, KDM1A expression was mainly distributed in malignant cells in HNSCC tumor tissues, highlighting that Pulrodemstat might selectively act on malignant cells to avoid potential side effects (Fig. 4D, E). In conclusion, our data demonstrate that KDM1A could be an attractive therapeutic target for HNSCC and Pulrodemstat might be a selective and efficient novel drug for HNSCC.

TET3 mediates the aberrant expression of KDM1A

Next, we elucidated the molecular mechanisms underlying aberrant KDM1A expression in HNSCC. The DNA methylation of CpG islands suppresses gene expression (ref). Interestingly, we found that methylation occupancy

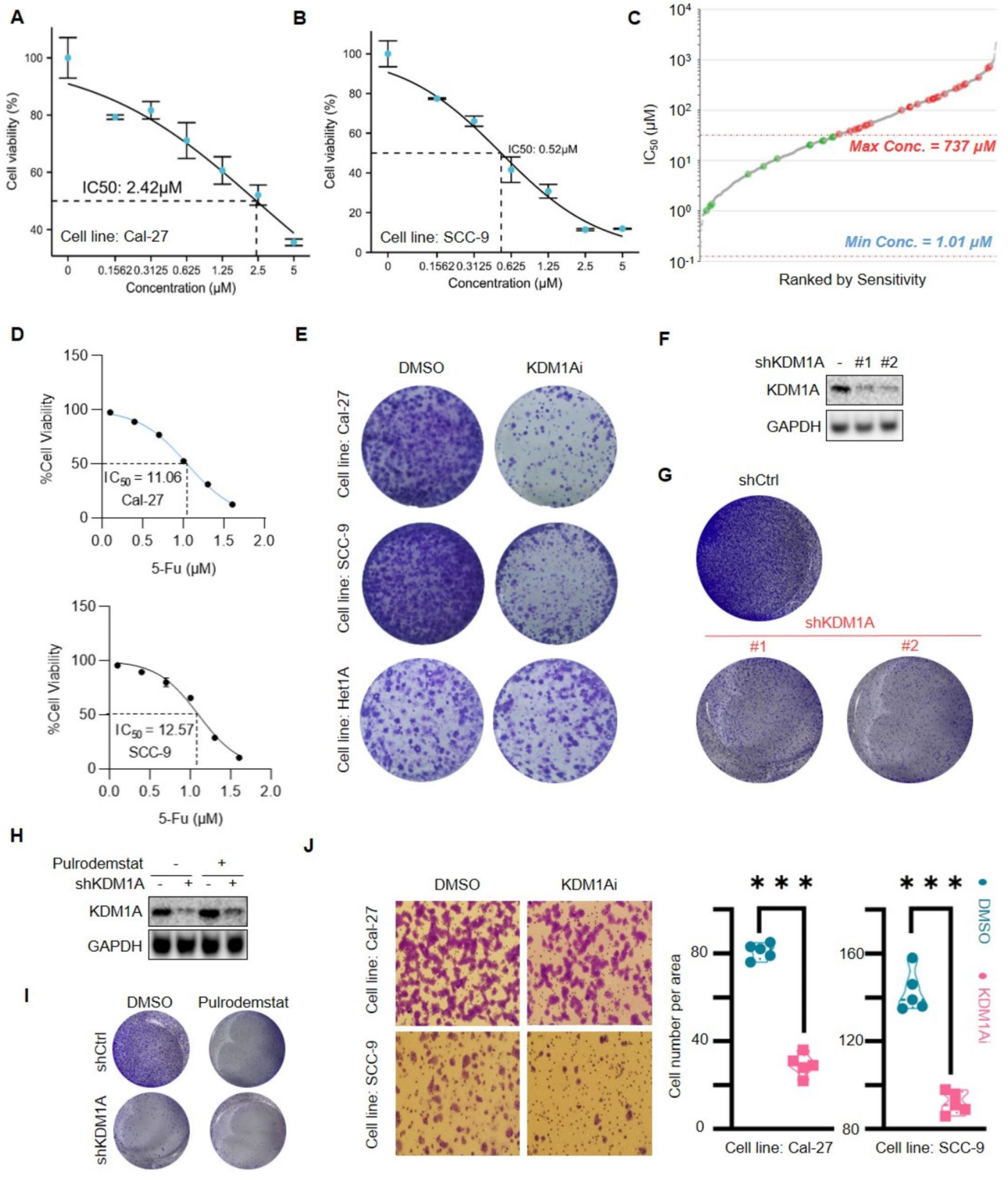

Fig. 2 Pulrodemstat suppresses HNSCC progress. (**A**, **B**) The IC_{50} of Pulrodemstat in each HNSCC cell lines were determined by CCK-8 assay. (**C**) The IC_{50} across HNSCC cell lines were analyzed by Genomics of Drug Sensitivity in Cancer database. (**D**) The IC_{50} of 5-Fu in each HNSCC cell lines were determined by CCK-8 assay. (**E**) The anti-tumor efficacy of Pulrodemstat in HNSCC cell lines were evaluated by colony formation assay. (**F**) Cal-27 cells were infected with indicated lentiviral constructs, followed by IB analysis as indicated. (**G**) Clonogenic assay in KDM1A-depleted Cal-27 cells. (**H**, **I**) Cal-27 cells stably expressing control or KDM1A shRNA were treated with Pulrodemstat and then subjected to IB (**H**) and clonogenic assay (**I**) as indicated. (**J**) The anti-metastasis capacity of Pulrodemstat in HNSCC cell liens were determined by transwell assay. Data in (**J**) was displayed as mean ± SD. Statistical significance was determined using Student's *t*-test. ***$P < 0.001$

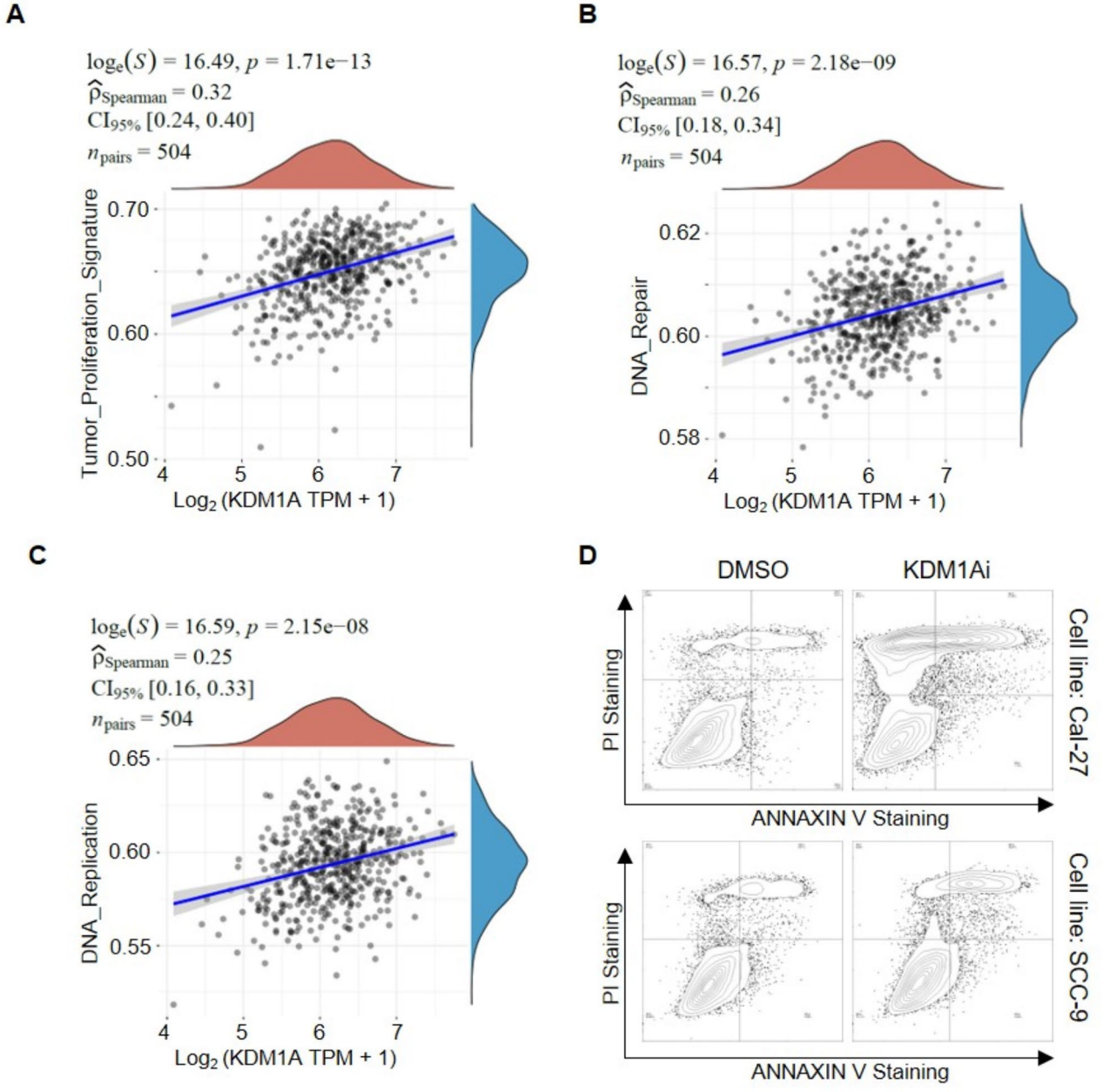

Fig. 3 Pulrodemstat blunts DNA repair and replication to induce apoptosis. (**A**) The correlation between KDM1A transcripts and tumor proliferation signature were determined by TCGA database. (**B**) The correlation between KDM1A transcripts and DNA repair signature were determined by TCGA database. (**C**) The correlation between KDM1A transcripts and DNA replication signature were determined by TCGA database. (**D**) The role of Pulrodemstat in regulating HNSCC cell lines apoptosis were evaluated by flow cytometry. The *p* values in (**A**–**C**) were conducted using R software (The R Foundation for Statistical Computing, 2020)

in the CpG islands of KDM1A was significantly lower in HNSCC tumor tissues than in normal tissues, indicating that the methylation status of CpG islands is involved in the regulation of KDM1A expression in HNSCC (Fig. 5A). Consistent with the relationship between KDM1A expression and clinical outcomes in HNSCC, we found that low methylation of KDM1A predicted poor clinical outcomes, as evidenced by shortened overall and progression-free survival times (Fig. 5B, C). In contrast, the methylation levels of KDM1A negatively correlated with its expression, suggesting that the aberrantly upregulated KDM1A levels in HNSCC might result from the loss of methylated CpG islands (Fig. 5D). More importantly, we found that the DNA demethylase, TET3, was positively associated with KDM1A expression, indicating that TET3 may contribute to KDM1A aberrant induction

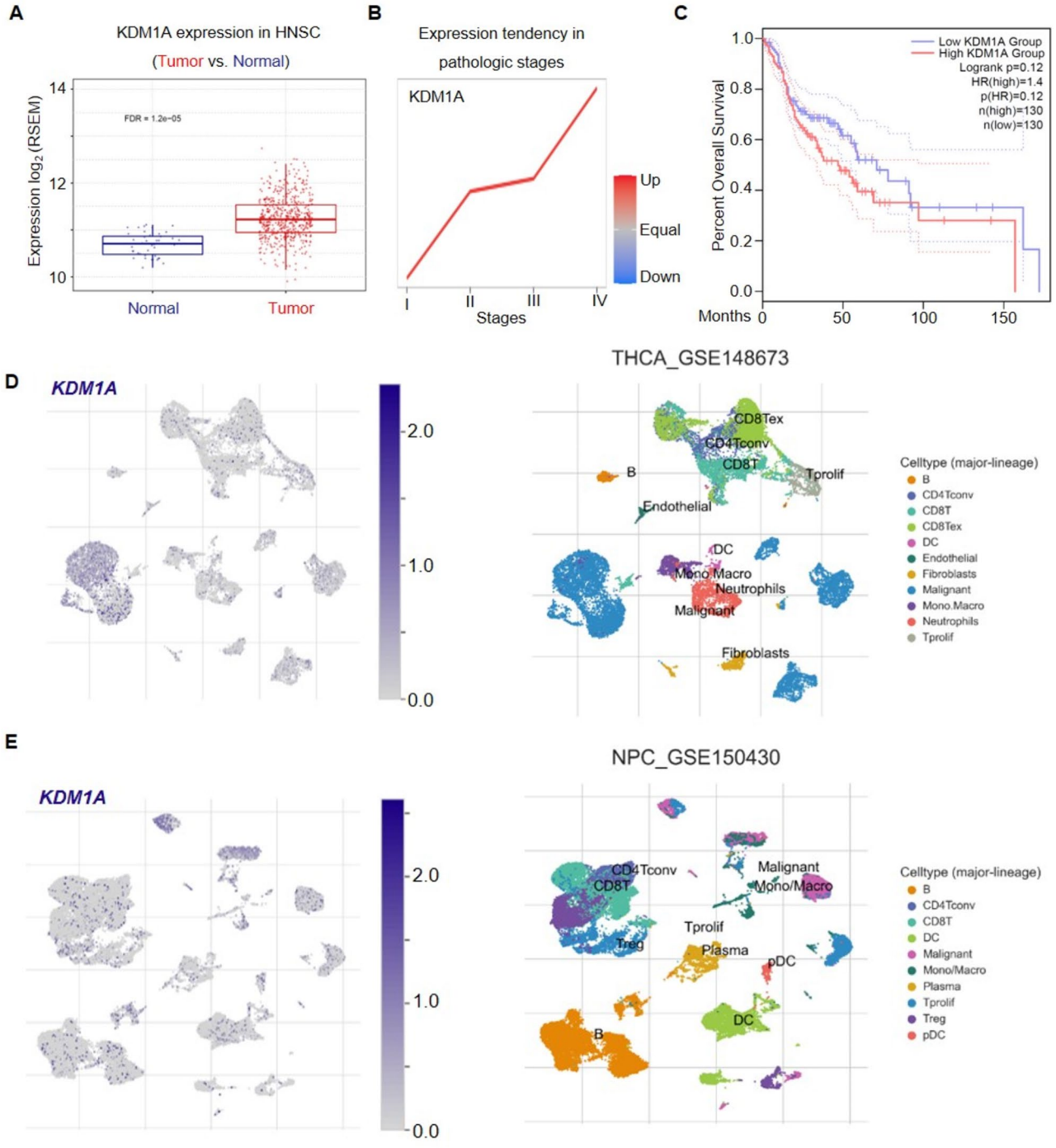

Fig. 4 The aberrant upregulation of KDM1A mainly distributed in HNSCC malignant cells. (**A**) The expression of KDM1A in HNSCC was analyzed by TCGA database. The significance of two sample groups is determined by the Wilcoxon test. (**B**) The correlation between KDM1A transcripts and HNSCC malignancies were determined by TCGA database. (**C**) The predictable value of KDM1A in HNSCC was determined by TCGA database via GEPIA online tools. All analytical methods above and R packages were performed using R software version v4.0.3 (The R Foundation for Statistical Computing, 2020). $p < 0.05$ was considered as statistically significant. (**D**, **E**) The expression distribution of KDM1A in HNSCC tumors were determined by single cell RNA-sequencing via TISCH2 database

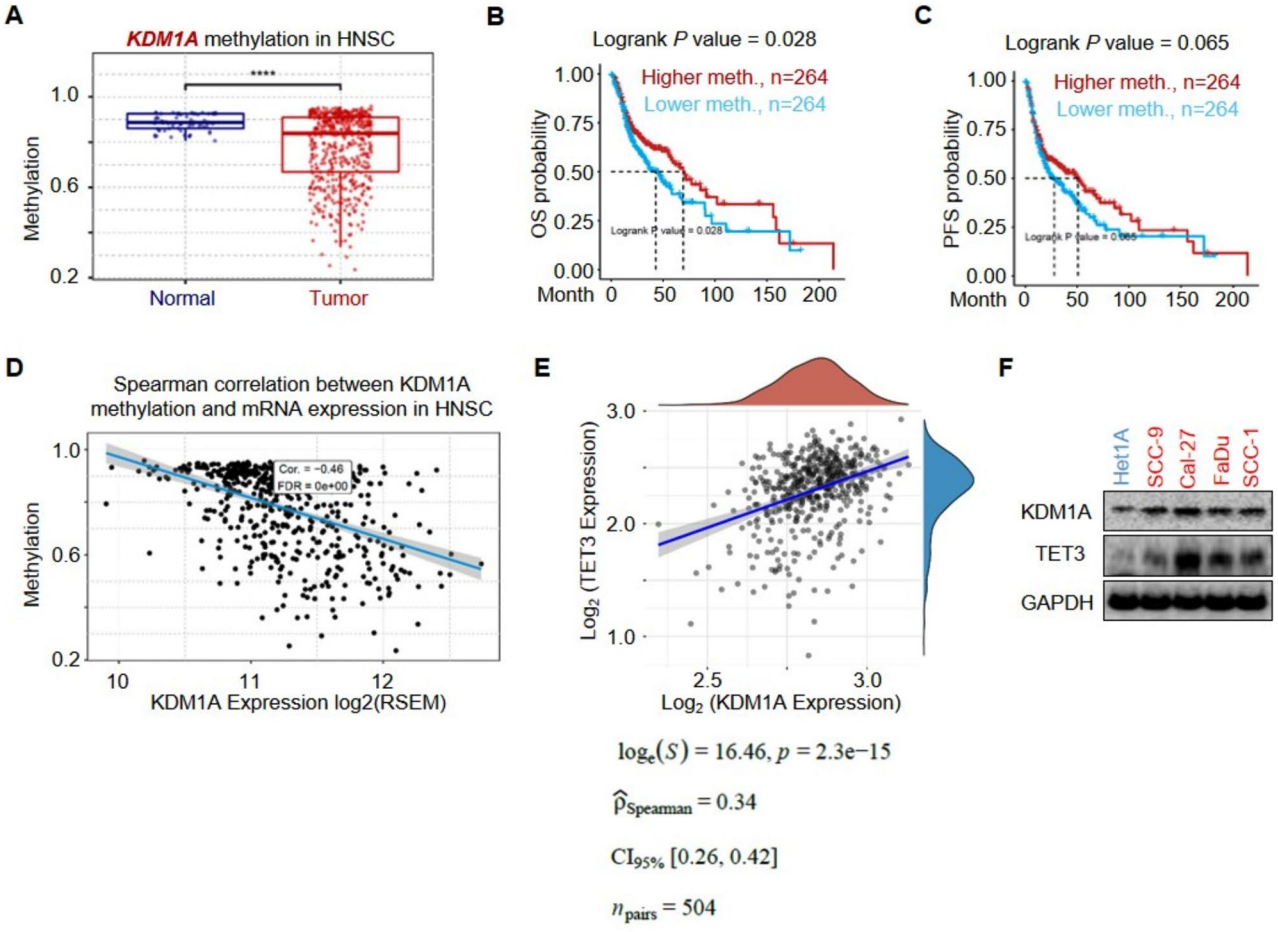

Fig. 5 TET3 serves as the upstream regulator of KDM1A. (**A**) The methylation levels of KDM1A in HNSCC was analyzed by TCGA database. (**B**, **C**) The correlation between KDM1A methylation levels and HNSCC clinical outcome were determined by TCGA database. (**D**) The correlation between LSD methylation levels and KDM1A transcripts were evaluated by TCGA database. (**E**) The correlation between TET3 and KDM1A were determined by TCGA database. (**F**) Hel1A, SCC-9, Cal-27, SCC-1 and FaDu cells were lysed and then subjected to IB analysis as indicated. Statistical analysis was conducted using R software, version v4.0.3. Results were considered statistically significant when the *p*-value was less than 0.05

by maintaining low methylation levels in the CpG islands of KDM1A in HNSCC (Fig. 5E). This observation was further confirmed in Het1A cells and the four HNSCC cell lines (Fig. 5F). Pulrodemstat treatment did not affect the expression levels of TET3, suggesting that KDM1A is not involved in the regulation of TET3. Collectively, our data revealed that TET3 may account for the aberrantly high expression of KDM1A in HNSCC by silencing DNA methylation markers.

Discussion

The number of patients with HNSCC is increasing annually, and the therapeutic regimen is still dominated by nonselective chemotherapeutic agents, which are prone to side effects and drug resistance [1]. Hence, there is an urgent need for precise and effective drugs. Accumulating evidence suggests that epigenetic dysregulation is a hallmark of cancer and several epigenetic modifier inhibitors are currently undergoing clinical trials [3, 5]. Therefore, we performed high-throughput drug screening to identify novel anti-HNSCC drugs. Fortunately, we found that Pulrodemstat, an ongoing phase II clinical trial for both hematologic and solid tumors, exhibited much stronger anti-HNSCC activity in vitro.

Pulrodemstat is an orally administered selective inhibitor of KDM1A [18]. KDM1A participates in the regulation of diverse biological processes through dynamic regulation of H3K4me2 and H3K9me2 demethylation [19]. In normal cell, KDM1A is reported to regulate neuronal differentiation [20]. More importantly, KDM1A is often found to be aberrantly upregulated across human cancers and serves as an oncogene to promote tumor progression, drug resistance and distant metastasis across human cancers [13, 21–24]. Therefore, KDM1A is an attractive therapeutic target for various cancer types [25]. To date, several KDM1A inhibitors have been developed [26, 27]. Inspiring, some of them are now ongoing clinical trials [7]. Of note, Pulrodemstat is the only

KDM1A inhibitors in clinical trials for the treatment of solid tumors, highlighting that our findings are promising for HNSCC therapy [28]. In addition, we found that KDM1A was dramatically upregulated in HNSCC tissues compared to normal tissues. Notably, scRNA sequencing revealed that KDM1A was mainly distributed in the malignant cluster, indicating that Pulrodemstat may be a powerful and selective therapeutic treatment for HNSCC.

The crosstalk between DNA and histone demethylation is an interesting regulatory model in dynamic biological processes [29]. For example, TET3, a DNA demethylase, has been reported to regulate neural commitment by controlling the expression of the H3K27me3 demethylase KDM6B by removing methylation marks in its CpG islands [9]. Interestingly, we found that the methylation levels of KDM1A tended to be lower in HNSCC samples than in normal tissues, which was consistent with the higher transcript levels of KDM1A. This suggests that low DNA methylation status might be the culprit for the aberrant upregulation of KDM1A and inspired us to hypothesize that DNA demethylases may be the upstream regulators of KDM1A. Consistent with our hypothesis, TET3 was positively associated with KDM1A expression levels in HNSCC samples.

Taken together, our investigation identified a promising anti-HNSCC drug that may be more efficient and selective and demonstrated that Pulrodemstat suppressed HNSCC progression by inducing apoptosis via disruption of the DNA replication and repair process. In addition, our analysis revealed an interesting regulatory network, the crosstalk between DNA and histone demethylation, in which TET3 may be responsible for the aberrant upregulation of KDM1A.

Conclusion

In conclusion, our study deciphered Pulrodemstat is a potent and selective anti-HNSCC drug. Pulrodemstat may blunt DNA repair and replication processes by impairing the catalytic activity of KDM1A. Moreover, KDM1A was aberrantly upregulated in HNSCC tissues and negatively associated with clinical outcomes. Notably, single-cell analysis revealed that KDM1A was mainly distributed in malignant cells, indicating the selective potency of Pulrodemstat. Moreover, we found that TET3 may contribute to the high expression of KDM1A in HNSCC tissues.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40360-024-00807-w.

Supplementary Material 1 Fig. S1 Determination of the IC50 of Pulrodemstat. (A–C) FaDu (A), SCC-1 (B) and Het1a cells were treated with indicated dosages of Pulrodemstat and then subjected to CCK-8 assay as indicated

Supplementary Material 2 Fig. S2 Pulrodemstat fails to alter the expression of TET3. Cal-27 cells were treated with gradient doses of Pulrodemstat, followed by IB analysis as indicated

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Author contributions
C.J. performed all in vitro experiments. The bioinformatics analysis was performed by X.W. J.Y. and Y.C. supervised the study. This manuscript was written by J.Y. and revised by Y.C.

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Data availability
The datasets generated and analyzed in the current study are listed in the Materials section. Data supporting the findings of this research and all resources used in this study can be requested from the corresponding authors.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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