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Targeting Ubiquitin-Specific Protease 14 Reduces Metastatic Potential and Metabolic Activity in Cervical Cancer via Direct Modulation of Monocarboxylate Transporter-4

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Abstract

Background: Cervical cancer (CC) remains a major health burden in low- and middle-income countries, where HPV vaccination coverage is suboptimal. Ubiquitin-specific peptidase 14 (USP14), a proteasome-associated deubiquitinase, has emerged as a potential driver of tumorigenesis, but its role in CC and impact on tumor metabolism remain poorly defined.

Methods:*In-silico* analysis of RNA and proteomics data from publicly available datasets was performed to assess the expression and prognostic value of USP14. Expression was validated in CC cell lines (HeLa, SiHa, CaSki) in comparison to non-transformed MCF10A cells. Functional studies involved USP14 knockdown (siRNA) or inhibition (IU1), followed by various cell viability, metastasis and invasion assays. Comparative proteomics was employed to identify USP14 interacting partners , with a particular focus on monocarboxylate transporter 4 (MCT4). USP14-MCT4 interactions were confirmed by co-immunoprecipitation, ubiquitination assays, and molecular dynamics simulations. Immunohistochemistry was performed on retrospective cervical squamous cell carcinoma tissues to correlate USP14 and MCT4 expression

Results: High USP14 expression correlated with poor overall survival in CC patients and was elevated in CC cell lines. Genetic or pharmacological inhibition of USP14 significantly reduced CC cell proliferation, metastasis, and invasion. . Proteomic profiling identified MCT4, a key lactate transporter in tumor metabolism, as a novel USP14 interactor. USP14 knockdown decreased MCT4 protein levels and stability, suggesting deubiquitination-dependent regulation. Immunoprecipitation confirmed direct binding between USP14 and

MCT4, and molecular dynamics simulations revealed distinct binding interfaces. In patient tissues, USP14 and MCT4 expression were positively correlated.

Conclusion: This study uncovers MCT4 as a novel substrate of USP14, linking USP14 activity to metabolic reprogramming in CC. The USP14MCT4 axis represents a previously unrecognized oncogenic pathway with therapeutic potential. Targeting USP14 could simultaneously impair tumor growth and disrupt lactate metabolism, offering a promising strategy for CC treatment.

Keywords- Ubiquitin-specific peptidase 14, Monocarboxylate transporter 4, Cervical Cancer, Ubiquitin, Metabolism, Cancer Metastasis

1. Introduction:

Cervical cancer (CC) remains one of the most prevalent gynaecologic malignancies and a significant global health challenge. According to GLOBOCAN 2022 data, it ranks as the fourth most common cancer among females and eighth overall, with a global age-standardized incidence rate (ASR) of 14.1 per 100,000 women. Asia bears a significant burden, with 60% of the global cases and 57.3% of deaths, and in India alone, CC is the second most common cancer among women.

Persistent infection with high-risk HPV types, especially HPV-16 and HPV-18, remains the main cause of CC globally, as it can lead to cervical intraepithelial neoplasia (CIN), a precancerous condition that may progress to invasive cancer if left untreated. The HPV genome encodes early(E1-E7) and late (L1-L2) proteins involved in replication, immune evasion, and viral structures (1,2).

CC screening has evolved from the traditional Pap smear to advanced molecular and imaging methods, including liquid-based cytology, HPV DNA testing, and RNA-based assays. Treatment approaches are stage-dependent and include surgery, radiation, chemotherapy, and immunotherapy, with new options offering more personalized approaches (3,4). The efficacy and side effects of these approaches are the crucial factors that significantly impact therapeutic outcomes. HPV vaccination using prophylactic vaccines such as Gardasil and Cervarix also serves as a critical preventive strategy in CC by inducing immune responses against high-risk HPV types (5).

Despite advancements in targeted therapies and immunotherapy, tumor heterogeneity continues to give rise to treatment-resistant clones, posing a significant hurdle in CC

management. This highlights the urgent need to understand the underlying mechanisms of resistance in the context of CC. The ubiquitin-proteasome system (UPS), an important regulatory mechanism for protein degradation, has drawn interest recently and emerged as a potentially effective cancer treatment option (6). The ubiquitin-proteasome system regulates protein fate through a coordinated process of ubiquitination, which is mediated by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3s), which act in tandem to regulate this important biological phenomenon. This process is reversed by deubiquitinating enzymes (DUBs), which stabilize the different oncoproteins and are increasingly recognized as key players in cancer progression and potential therapeutic targets (7). Among DUBs, ubiquitin-specific proteases (USPs) are the largest and most studied family as they are crucial regulators of oncogenes and tumour suppressor genes, significantly influencing cancer progression (8,9).

Studies have described the interplay of one of the most studied member of Ubiquitin-specific proteases (USPs) family such as USP7 in DNA damage repair, replication, and tumor progression through deubiquitination. (10). Recent Studies also suggested that other USPs likeUSP10(11), USP37 (12–14), USP35 (15), plays critical roles in cancer by stabilizing oncogenic proteins, regulating the cell cycle progression, promoting epithelial-mesenchymal transition (EMT), metastasis, and resistance to replication stress, thus contributing to tumor progression and representing promising therapeutic targets.

USP14, another member of the deubiquitinase family, plays an essential oncogenic role in various cancers by stabilizing key regulatory proteins and modulating signalling pathways that control cell proliferation, apoptosis, and treatment resistance (1617,18). In breast cancer, USP14 is overexpressed and is associated with poor prognosis and tumor progression through cyclin B1 and androgen receptor signalling and maintaining CDK1 stability, thereby promoting uncontrolled proliferation and cell cycle progression. (19,2021)

USP14 plays a critical role in promoting tumor growth and therapy resistance across multiple cancer types through diverse molecular pathways. In hepatocellular carcinoma, USP14 enhances tumor development by activating the Wnt/β-catenin and PI3K/Akt signaling pathways.(22,23). USP14 also contributes to epithelial ovarian cancer progression and cisplatin resistance via BCL6 stabilization (24). In colorectal cancer, USP14 stabilizes IDO1, leading to increased tryptophan metabolism and immune suppression(25). In pancreatic ductal adenocarcinoma**,** USP14 forms a self-amplifying loop with TAZ, stabilizing it to drive

tumor growth and liver metastasis (26). In endometrial cancer**,** USP14 supports tumor growth by stabilizing estrogen receptor α (ERα), correlating with poor prognosis and offering a target for hormone-sensitive tumors (27). These findings highlight the oncogenic roles of USP14 across multiple cancers, though its function in cervical cancer development remains poorly understood. This study addresses this gap by integrating *in-silico*, *in-vitro*, and archived patient tissue analyses to evaluate the expression and functional role of USP14 in cervical cancer progression. This study identified MCT4 as a novel binding partner of USP14, demonstrating that USP14 promotes cervical cancer oncogenesis by deubiquitinating and stabilizing MCT4, leading to altered lactate efflux and reprogrammed cellular metabolism. These findings provide new insights into the oncogenic network of USP14 in cervical cancer.

2. Materials and methods:

2.1 Cell Culture

The mammary epithelial cell line MCF10A was obtained from Elabscience and cultured in DMEM/F12 medium (Thermo Fisher Scientific, Cat. No. 11320033) supplemented with 5% horse serum (Thermo Fisher Scientific, Cat. No. 26050070), 20 ng/mL epidermal growth factor (EGF; Gen Script, Cat. No. Z00333), 10 µg/mL insulin (Elabscience, Cat. No. PB180432), 0.5 µg/mL hydrocortisone (Sigma, Cat. No. H0135), and 100 ng/mL cholera toxin (Sigma, Cat. No. C3062). CC cell lines HeLa, SiHa, CaSki, ME180, and C33A were procured from the National Centre for Cell Science (NCCS), Pune. HeLa, SiHa, and ME180 cells were cultured in high-glucose DMEM (Thermo Fisher Scientific, Cat. No. 11965092) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Cat. No. 10270106) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Cat. No. 15140148). C33A cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine (Thermo Fisher Scientific, Cat. No. 25030149), and 1% non-essential amino acids (Thermo Fisher Scientific, Cat. No. 11140050). CaSki cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Cat. No. 11875093) supplemented with 2 mM L-glutamine and 10% FBS. All the cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Culture media was changed every 2–3 days, and cells were routinely passaged upon reaching approximately 80% confluence.

2.2 Drug Preparation and Treatment

Cisplatin (Cat. No.: HY-17394, MedChemExpress), was dissolved in sterile distilled water at a stock concentration of 1mg/mL (3.33mM). This stock solution was further diluted to 20µM and 40µM in culture media to prepare working standards for cell treatment. 10 mM stock solution of deubiquitinase inhibitor (IU1: Cat. No.: HY-13817, MedChemExpress), was prepared and diluted to make working standards of 50µM and 100µM in cell culture media for *in vitro* experiments. 10mg/mL stock solution of Cycloheximide (Cat. No.: #2112, Cell Signaling Technology) was prepared by dissolving 50mg of powder in 5mL of DMSO and further diluted to a final concentration of 100µg/mL. All drug treatments were performed under sterile conditions, and appropriate vehicle controls were included in all the experiments.

2.3 *In Silico* Analysis of USP14 Expression in Cervical Cancer: Transcriptomic, Proteomic, and Survival Correlation Approaches

RNA sequencing data from The Cancer Genome Atlas (TCGA) were analyzed using the GEPIA2 platform (http://gepia2.cancer-pku.cn/). The pathological stage plot feature was used to examine USP14 expression across different clinical stages (I–IV) of cervical cancer, and Kaplan–Meier survival analyses for overall survival (OS) and disease-free survival (DFS) were performed by stratifying patients into high and low USP14 expression groups. To assess USP14 mRNA expression in cervical cancer cell lines, transcriptomic data were retrieved from the Cancer Cell Line Encyclopedia (CCLE) and expressed as Transcripts Per Million (TPM), which were visualized as bubble plots using R. Corresponding protein expression levels were obtained from the Cell Model Passports proteomic database (https://cellmodelpassports.sanger.ac.uk) and similarly represented as bubble plots to illustrate differential USP14 protein expression across cell lines.

2.4 Gene Expression Analysis by Quantitative Real-Time PCR

Real-time qPCR was done to evaluate the relative gene expression of the specific gene USP14. Total RNA was extracted from SiHa and HeLa cells using TRIzol™ reagent (Thermo Fisher Scientific, Cat. No. 15596026) and reverse transcribed into cDNA with the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. AB1453A). Quantitative real-time PCR

was carried out on the LightCycler® 480 system using SYBR Green I Master Mix and gene-specific primers for the specific gene USP14. β-actin served as the internal control. The forward and reverse primers are detailed in Table.1 of Supplementary file 3. Reactions were run in triplicate, and relative expression analysis by fold change was calculated using the ΔΔCt method.

2.5 **Transfection:**

HeLa and SiHa cells were transfected with USP14-specific siRNA (Santa Cruz Biotechnology, sc-76817) at concentrations of 50 and 100 pmol using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Cat. No. 13778150) according to the standard protocols. A non-targeting siRNA (Control siRNA-A, sc-37007) was used as a negative control. Plasmids including GFP-Ub (Addgene #11928), HA-Ub (Addgene #18712), and His-USP14 (GenScript) were cultured, amplified, and purified via miniprep kit (cat. nos. 27104, Qiagen). Plasmid transfections were performed using Lipofectamine 3000 (Cat. No. L3000001) with Opti-MEM (Thermo Fisher Scientific, Cat. No. 31985070) for DNA-lipid complex formation. Cells at 70–90% confluency were transfected and incubated under standard conditions, with assays conducted 4h post-transfection.

2.6 **Colony Formation Assay**

Colony formation assay was conducted using HeLa and SiHa cell lines under three experimental conditions: **(**i) Cells were transfected with USP14-targeting siRNA and control siRNA-A. At 48h post-transfection, cells were trypsinized and seeded into 6-well plates at a density of 500–1000 cells per well. Cells were maintained for 10-15 days, with media changes every 2-3 days. **(**ii) Cells were transfected as described above, trypsinized after 48h, and seeded at 500-1000 cells per well. After 24h, cells were treated with 20μM or 40μM cisplatin for 24h, followed by replacement with fresh medium. Colonies were allowed to form over 10–14 days with regular media replacement. **(**iii) HeLa and SiHa cells were seeded at 500–1000 cells per well and treated with 50μM or 100μM IU1 for 24h after attachment. The medium was then refreshed, and the cells were incubated for 10–14 days with periodic media changes. At the end of the incubation period, colonies were fixed with 4% paraformaldehyde (Sigma-Aldrich, Cat. No. P6148) for 15 minutes and stained with 0.5%

crystal violet in 25% methanol (Sigma-Aldrich, Cat. No. C0775) for 30 minutes. Excess stain was removed with distilled water, and plates were air-dried. Colonies were quantified, and the counts were normalized to the Si Control group. All the conditions were tested in triplicate, and data were expressed as mean ± standard deviation (SD) from three independent experiments.

2.7 Scratch Wound Healing Assay

Scratch wound healing assays were performed on HeLa and SiHa cells under two conditions to assess cell migration. In the first group, the cells were transfected with USP14-specific and control siRNA. After 48h, the cells were seeded into 24-well plates, and a scratch was made using a 200 µL tip once they reached near confluency. Images were captured at 0 and 24h post-scratch. In the second group, both cells were seeded similarly, scratched after 24h, and treated with 50 µM or 100 µM IU1 (USP14 inhibitor) following a PBS wash. Wound closure was monitored at 0 and 24h. Experiments were done in triplicate, and migration was quantified using ImageJ.

2.8 Transwell Migration Assay

Transwell migration assays were performed in CC cells (HeLa and SiHa) under USP14 knockdown and pharmacological inhibition conditions. Cell migration was evaluated using Transwell inserts with 8.0µm pore size polycarbonate membranes (Corning, Cat. No. CLS3399) fitted into 24-well culture plates. For the siRNA-mediated knockdown experiments, HeLa and SiHa cells were transfected with USP14-specific siRNA and non-targeting control siRNA. After 48h, the transfected cells were trypsinized, counted, and resuspended in 1% FBS-containing DMEM. A total of 1×10^5 cells in 500 µL of this medium was added to the upper chamber of each insert, while the lower chamber was filled with 600 µL of complete DMEM supplemented with 10% FBS to serve as a chemoattractant. The cells were incubated at 37°C in a humidified CO_2 incubator for 24h. For pharmacological inhibition, around 1×10^5 HeLa and SiHa cells were seeded into the upper chambers in 600 µL of 1% FBS-containing medium supplemented with IU1 (USP14 inhibitor at final concentrations of 50µM or 100µM. The lower chambers were filled with 10% FBS-containing medium as described above. After 24h of incubation, non-migrated cells on the

upper membrane surface were gently removed using a cotton swab. Migrated cells on the underside of the membrane were fixed with 4% paraformaldehyde for 15 minutes at room temperature and stained with 0.5% crystal violet for 30 minutes. Inserts were then washed thoroughly with distilled water, air-dried, and imaged using a bright-field microscope. Migrated cells were quantified manually in randomly selected fields per insert or analyzed using ImageJ software. All experiments were conducted in triplicate, and data are presented as the mean ± standard deviation (SD) from three independent biological replicates.

2.9 Proteomic Profiling and Data Analysis:

SiHa cells were transfected with siRNA targeting USP14 and a non-targeting siRNA control for 48h. Post-transfection, the cells were harvested, washed twice with PBS, and processed for proteomics analysis as described previously (28).

Cells were lysed in RIPA buffer and proteins precipitated with chilled acetone at −20°C overnight. Pellets were resuspended in 8 M urea (Tris-HCl, pH 8.5), quantified by Bradford assay, reduced with DTT, alkylated with IAA, and digested with trypsin overnight at 37°C. Peptides were purified using Oasis HLB cartridges, dried, and stored at -20°C. For SWATH-MS, peptides were analyzed on a TripleTOF 6600 (SCIEX) coupled with an Eksigent NanoLC-425 system using a 57-min linear gradient. Data were acquired in SWATH mode with 100 variable windows and processed in Spectronaut v19 (directDIA workflow) using the UniProtKB human database with 1% FDR. Dysregulated proteins were identified based on fold change, and results visualized using R (volcano plots) and Excel (heatmaps). Functional enrichment was performed via Reactome and GO analyses to identify key pathways and biological processes. Relative proportion of buffers used in SWATH-MS is mentioned in table 2 of supplementary file 3.

2.10 Molecular Docking and Molecular Dynamics Simulations

). The full-length structure of MCT4 (UniProt ID: SLC16A3) was obtained from AlphaFold, (29)while the catalytic domain of USP14 was retrieved from the PDB (ID: 6IIK). Molecular docking between MCT4 and USP14 was performed using the ClusPro server, and the top-

ranked complex with the lowest energy and largest cluster size was selected for 200 ns MD simulation using GROMACS 5.0 to assess structural stability. (30,31,32)

For the MD simulations, the GROMOS54a7 force field was used (33) and the complex was placed in a cubic box of 10 Å, followed by solvation using the water SPC216 model (34), energy minimization (using steepest descent algorithm with < 1000.0 kJ/mol/nm convergent criteria) and equilibration at 300 K. Thereafter, both NVT and NPT calculations were conducted for the USP14-MCT4 complex, followed by 200 ns MD simulations using the same detailed protocol as in our previous studies (12,28). Finally, the Root mean square fluctuation (RMSF), Root mean square deviation (RMSD), Radius of gyration (Rg) and Solvent accessible surface area (SASA), were calculated, and the plots were created using SigmaPlot 10. The structural figures were created using Pymol. To show the stability of the interactions between the USP14 and MCT4, Contact Maps were plotted for 0 and 200 ns frames using the Mapiya Contact Map server by employing default parameters (35). To support the Contact map finding, the Pubsum server was used to identify the interactions between the USP14-MCT4 complex. Additionally, the GPS-uber server was used to identify the possible ubiquitination sites in the MCT4 (36).

2.11 Immunoblotting

USP14 expression was examined in CC cell lines (HeLa, SiHa, CaSki, C33A, ME-180) and the non-tumorigenic epithelial line MCF10A. Cells were lysed in RIPA buffer with protease and phosphatase inhibitors, and protein concentrations were determined using the BCA assay. Equal protein amounts were resolved by SDS-PAGE and transferred onto PVDF membranes. For USP14 knockdown, HeLa and SiHa cells were transfected with siUSP14 (50–100 pmol) or siControl for 48 h, and for pharmacological inhibition, cells were treated with IU1 (50 μM and 100 μM) for 24 h before lysis. Membranes were blocked with 5% BSA, incubated overnight at 4°C with primary antibodies against USP14, β-actin, E-cadherin, N-cadherin, Vimentin, MMP2, MMP9, and MCT4 (1:1000–1:2000), followed by HRP-conjugated secondary antibodies (1:5000). Bands were detected using ECL reagent and imaged on a Bio-Rad ChemiDoc system. Densitometric analysis was performed using ImageJ, and expression levels were normalized to β-actin across three independent experiments.

2.12 Immunoprecipitation

To assess the endogenous interaction between USP14 and MCT4 in SiHa CC cells, both forward and reverse immunoprecipitation (IP) assays were performed. SiHa cells were lysed in IP lysis buffer (Thermo Fisher Scientific, Cat. No. 87787) supplemented with protease and phosphatase inhibitors. The total protein concentration was measured using the BCA assay. Equal amounts of protein (1mg per condition) were used for each IP setup. For forward IP, the lysates were pre-cleared with 20µL of Protein A/G agarose beads (Santa Cruz, sc-2003) at 4°C for 3–4h. Cleared lysates were incubated overnight with an anti-USP14 antibody (5–10µg per 500- 1000 µg protein) or a mouse IgG isotype control (2 µg per 1000 µg protein) (Thermo Fisher Scientific, Mouse IgG Isotype Control, 31903). The next day, 20 µL of fresh Protein A/G beads were added and rotated for 2–3h at 4°C to capture the complexes. Beads were pelleted (10,000 rpm, 10 min) and washed three times with cold IP buffer. For reverse IP, a similar procedure was followed using anti-MCT4 antibody and an IgG control. Following the final wash, bound proteins were eluted by boiling the beads in 4× Laemmli buffer with 5% β-mercaptoethanol at 95°C for 10 minutes. After centrifugation, the eluates and 5% input controls were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% BSA in PBST and probed overnight with anti-MCT4 (forward IP) or anti-USP14 (reverse IP) antibodies. Detection was carried out using HRP-conjugated secondary antibodies and enhanced chemiluminescence.

2.13 Deubiquitination Assay

To determine whether USP14 deubiquitinates MCT4, SiHa cells were used in both knockdown and overexpression settings. For knockdown, the cells were seeded and co-transfected with siControl + GFP-ubiquitin (GFP-Ub) and si-USP14 + GFP-Ub plasmids using Lipofectamine, followed by 48h incubation. In the overexpression setup, SiHa cells were co-transfected with HA-Ub + His-USP14 and HA-Ub + empty vector for 48h. After incubation, the cells were harvested, washed with PBS, and lysed in IP lysis buffer (1mL per sample) containing protease and phosphatase inhibitors. Lysates were clarified by centrifugation (15,000 rpm, 15 min, 4°C), and protein concentrations were determined using the BCA assay. Each sample (500µg protein) was pre-cleared with 20µL of Protein A/G agarose beads at 4°C for 3–4h, then incubated overnight at 4°C with anti-MCT4 (5- 10 µg per 500- 1000 µg protein) antibody under gentle rotation. The next day, 20µL of fresh Protein

A/G beads were added to capture immune complexes and incubated for 3–4h at 4°C. Beads were washed three times with cold IP lysis buffer and eluted by boiling in 4× Laemmli buffer at 95°C for 10 minutes. Eluates were subjected to SDS-PAGE, transferred to PVDF membranes, and blocked in 5% BSA/PBST. For the knockdown experiments, the membranes were probed with anti-GFP antibody to detect ubiquitinated MCT-4. For the overexpression condition, an anti-HA antibody was used. Detection was performed using HRP-conjugated secondary antibodies and enhanced chemiluminescence. To validate USP14 knockdown and equal protein loading, input lysates were separately immunoblotted with anti-USP14 and anti-β-actin antibodies as per the dilutions used in immunoblotting.

2.14 Cycloheximide Chase Assay

To assess the impact of USP14 inhibition on MCT4 stability, a cycloheximide (CHX) chase assay was performed in SiHa cells. Cells were transfected with siControl and si-USP14 (50 pmol) and incubated for 48h. Post-transfection, the cells were reseeded and treated with CHX (100µg/mL) to block protein synthesis. Samples were collected at 0, 2, 4, and 8h, lysed in RIPA buffer with protease/phosphatase inhibitors, and the protein levels were quantified via BCA assay. Equal protein amounts were analyzed by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against USP14, MCT4, and β-Actin as per dilutions used in immunoblotting. Detection was performed using HRP-conjugated secondary antibodies and ECL reagents.

2.15 Lactic Acid Estimation Assay

To assess the impact of USP14 on extracellular lactate levels, lactic acid measurements were performed in SiHa and HeLa cells following USP14 knockdown and pharmacological inhibition. The cells were transfected with either siControl or si-USP14 for 48h. For inhibition, the cells were treated with IU1 (USP14 inhibitor) at concentrations of 50µM and 100µM for 24h. Following treatment, the culture supernatants were collected and centrifuged to remove cellular debris. Lactic acid levels were quantified using the L-Lactic Acid Colorimetric Assay Kit (E-BC-K044-S, Elabscience) according to the manufacturer's

instructions. Briefly, 20µL of each sample was incubated with the enzyme and chromogenic reagents at 37°C, followed by the addition of a stop solution. Absorbance was measured at 530nm using a microplate reader. All measurements were performed in triplicate. Lactic acid concentration was calculated using the formula:

Lactic acid (mmol/L) = (ΔA1 / ΔA2) × c × f, where ΔA1 = OD sample – OD blank, ΔA2 = OD standard – OD blank, c = standard concentration (3 mmol/L), and f = dilution factor of the sample.

2.16 Immunofluorescence Microscopy

To assess the impact of USP14 silencing on MCT4 expression and localization, SiHa cells were transfected with siControl or si-USP14 for 48 h and then seeded onto sterile chambered slides. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA. They were then incubated overnight at 4°C in a humidified chamber with primary antibodies against MCT4 (1:200) and USP14 (1:100), followed by Alexa Fluor® 488-conjugated secondary antibody (1:1000, Cat. No. A-11008). Nuclei were counterstained with DAPI and mounted using Fluoroshield™ (F6057). Fluorescence images were acquired using a Leica microscope at 40× and 60× magnifications under identical exposure settings to compare MCT4 localization and expression between the control and USP14-silenced cells.

2.17 Immunohistochemistry

To evaluate the expression patterns of USP14 and MCT4 in clinical cervical tissues, immunohistochemical analysis was performed on 6 normal cervical tissues and 30 CC samples. Archival formalin-fixed paraffin-embedded (FFPE) cervical squamous cell carcinoma (SCC) tissue samples with a histopathologically confirmed diagnosis and available clinical and follow-up data were included in the study. Only cases with no prior history of malignancy and well-preserved archived blocks suitable for IHC were considered. Samples were excluded if there was any history of previous malignancy, chemotherapy, or gene therapy, or if the tissues were poorly fixed or inadequately preserved for

immunohistochemical analysis. Patients details of archived tissues sections is mentioned in table 3 of supplementary file 3. Sections (4μm thickness) were mounted on positively charged slides, deparaffinized in xylene, and rehydrated through graded ethanol. Antigen retrieval was performed using Tris-EDTA buffer (pH 8.0) at 100°C for 30min, followed by quenching of endogenous peroxidase activity with 3% hydrogen peroxide. Non-specific binding was blocked using the UltraVision Quanto Detection System (HRP DAB, TL-060-QHD). Sections were then incubated overnight at 4°C with primary antibodies against USP14 and MCT4 (Santa Cruz Biotechnology; 1:100 dilution). After washing, the slides were incubated with HRP-conjugated goat anti-rabbit secondary antibody, and the signal was developed using DAB chromogen, resulting in a brown precipitate at antigen sites. Counterstaining was performed with hematoxylin, followed by dehydration and mounting with DPX. Placenta tissue (showing membranous staining) and stomach tissue (showing cytoplasmic staining) were used as positive controls for MCT4 and USP14, respectively. Evaluated the stained sections, and the expression was semi-quantitatively scored based on the percentage of positive cells (1: 0–25%, 2: 25–50%, 3: 50–75%, 4: 75–100%) and staining intensity (1: weak, 2: moderate, 3: strong). The final IHC score was calculated as the product of these two parameters. Comparative analysis was conducted to assess the differential expression of USP14 and MCT4 between normal and cancerous tissues. Representative images were captured for both markers, and the Pearson correlation coefficient was calculated to evaluate the relationship between their expression levels.

2.18 Data availability

The dataset created for the study titled “Targeting Ubiquitin-specific proteases (USP14) Reduces Metastatic Potential and Metabolic Activity in CC by Modulating MCT4” has been successfully submitted to ProteomeXchange through the PRIDE database. The mass spectrometry proteomics data are now available in the ProteomeXchange Consortium via the PRIDE partner repository, and can be accessed using the dataset identifier PXD067901. For further details regarding the submission, the project is named "Targeting Ubiquitin-specific proteases (USP14) Reduces Metastatic Potential and Metabolic Activity in CC by Modulating MCT4" with the project accession PXD067901 and the project DOI is not applicable and reference: 1-20250830-131918-2745934

2.19 Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Differences between the two groups were analyzed using an unpaired Student's *t*-test. For comparisons involving more than two groups, one-way or two-way ANOVA followed by appropriate post hoc tests was used, as indicated. A p-value of <0.05 was considered statistically significant.

3. Results

3.1 USP14 expression analysis in cervical cancer and its association with poor prognosis

To assess the expression of USP14 and its clinical significance in CC, we used the Gene Expression Profiling Interactive Analysis (GEPIA2) platform, which integrates data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. Single-gene expression analysis revealed that USP14 expression is significantly elevated in cervical tumor samples (n = 306) compared to normal tissues (n = 13), as shown in the box plot (Figure 1A). To further examine whether USP14 expression correlates with disease progression, we analyzed its expression across pathological stages (Stage I–IV) using a stage plot and a violin plot. The data showed stage-associated variation in USP14 expression, with the violin plot displaying the distribution, median, and interquartile range for each stage. (Figure S1A) These findings suggest that USP14 is not only upregulated in cervical tumors but also varies with disease progression. To evaluate the prognostic relevance of USP14, survival analysis was performed using GEPIA2. Kaplan–Meier curves, which indicated that high USP14 expression is significantly associated with poor overall survival (Figure 1B) and disease-free survival (Figure S1B) in CC patients.

These findings are related to the expression of USP14 in CC patients through *in-silico* analysis. Therefore, to further investigate the expression in cell lines, we analyzed USP14 expression at both the RNA and protein levels in CC cell lines using publicly available datasets. The mRNA expression data analysis was done using Cancer Cell Line

Encyclopaedia (CCLE), which indicated elevated USP14 levels in CC cell lines, including HeLa, SiHa, and ME180 (Figure 1C). Corresponding protein expression data in CC cells were extracted from the Cell Model Passports proteomic repository, confirming upregulated USP14 protein levels in the same cell lines (Figure 1D). To further determine whether USP14 expression is associated with CC cell transformation, we compared its expression in non-transformed epithelial cells (MCF10A) to that in various CC cell lines (HeLa, SiHa, CaSki, ME180, and C33A). Both RNA (Figure 1E) and protein (Figure 1F) analysis showed significantly higher USP14 expression in the cancer cell lines compared to MCF10A, suggesting a potential role for USP14 in CC oncogenesis. Collectively, in-silico analysis consistently demonstrates that USP14 is upregulated in CC tissues and cell lines at both transcript and protein levels, and its elevated expression correlates with poor prognosis and disease progression, supporting its potential role in CC development and progression.

Figure 1. USP14 expression analysis in cervical cancer and its association with poor prognosis. (**A**) Box plot showing USP14 mRNA expression levels in normal (n = 13) and cervical cancer tissues (n = 306) based on the TCGA dataset. Expression of USP14 is significantly upregulated in cervical cancer tissues. The red box represents tumor (CESC) samples, while the grey box represents normal tissues. Data are presented as $\log_2$ (TPM + 1) values, with the box indicating the interquartile range (IQR) and the central line representing the median expression level. (**B**) Kaplan–Meier survival analysis based on TCGA dataset demonstrating the correlation between USP14 expression levels and overall survival in cervical cancer patients. High USP14 expression is associated with poorer prognosis. Bubble plots representing USP14 mRNA (**C**) and protein (**D**) expression across various cervical cancer cell lines from publicly available datasets. USP14 is overexpressed in cervical cancer cell lines panel at RNA and protein level. (**E**) qRT-PCR analysis of USP14 mRNA expression in normal breast epithelial cell line (MCF-10A), and various cervical cancer cell lines (CaSki, C-33A, HeLa, SiHa, and ME-180). USP14 is significantly overexpressed in cervical cancer cell lines compared to MCF-10A. Data shown as mean ± SD (Statistical significance was assessed using one-way ANOVA), * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ns: not significant. (**F**) Western blot analysis showing USP14 protein expression in MCF-10A and cervical cancer cell lines. Densitometric quantification of USP14 normalized to Actin is

shown on the right. Increased USP14 protein levels were observed in all cervical cancer cell lines. Data shown as mean ± SD (Statistical significance was assessed using one-way ANOVA),* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ns: not significant

3.2 Inhibition of USP14 leads to reduced survival and metastasis in Cervical Carcinoma Cells

Recent studies have shown that USP14 inhibition suppresses cell proliferation and growth in breast and hepatocellular carcinoma (19,23). To further explore the role of USP14 inhibition in CC proliferation and growth, we performed colony formation assays in CC cells following USP14 knockdown using siRNA. The results revealed a significant reduction in colony numbers after the inhibition of USP14 in HeLa (Figure 2A) and SiHa cells (Figure 2B).

Another recent study has indicated that USP14 contributes to chemoresistance against platinum-based chemotherapy, such as cisplatin, in ovarian cancer (24). To explore the involvement of USP14 in regulating cisplatin sensitivity in CC cells, colony formation assays were carried out in HeLa and SiHa cells following USP14 knockdown and exposure to cisplatin. Our results indicate that USP14 inhibition significantly reduces colony formation ability at 20 μM and 40 μM cisplatin in both HeLa (Figure 2C) and SiHa cells (Figure 2D). These results indicate that USP14 inhibition enhances the chemosensitivity of both the CC cell lines.

Previous studies have also demonstrated that USP14 plays a role in regulating migration and metastatic potential in various cancer cell lines, including those of gastric cancer (37). Similarly, to explore the mechanistic role of USP14 in CC cell migration and metastasis, scratch wound healing assays were conducted in HeLa and SiHa cells following USP14 knockdown. The results showed a marked reduction in wound closure after 24h in both HeLa (Figure 2E) and SiHa (Figure 2F) cells, suggesting that USP14 inhibition significantly impairs the migratory capacity of CC cells. Additionally, transwell migration assays were conducted to assess the impact of USP14 on the migratory potential of CC cells. The results

showed that siRNA-mediated knockdown of USP14 led to a significant reduction in the number of migrated HeLa (Figure 2G) and SiHa (Figure 2H) cells, indicating its role in promoting cell migration.

Moreover, to further validate the role of USP14 in regulating epithelial–mesenchymal transition (EMT) in CC cells, the expression levels of key EMT markers, including E-cadherin, N-cadherin, Vimentin, MMP2, and MMP9, were analyzed following USP14 inhibition in SiHa cells. The results indicate that the USP14 inhibition led to a reduction in mesenchymal and metastatic markers (N-cadherin, Vimentin, MMP2, and MMP9), along with an upregulation of the epithelial marker E-cadherin, supporting the involvement of USP14 in EMT regulation (Figure S2A). Collectively, these findings highlight that USP14 regulates the CC cell proliferation, chemoresistance, migration, and EMT, suggesting its potential role in promoting metastasis.

Figure 2. Inhibition of USP14 leads to reduced survival and metastasis in cervical carcinoma cells. (**A–B**) Colony formation assay showing a significant reduction in clonogenic survival of HeLa (**A**) and SiHa (**B**) cells upon USP14 knockdown using siRNA. Quantification is shown on the right ($p < 0.01$, unpaired *t*-test). (**C–D**) Clonogenic survival assays in HeLa (**C**) and SiHa (**D**) cells treated with control siRNA or si-USP14, followed by increasing concentrations of cisplatin (0, 20 μM, and 40 μM). USP14 knockdown sensitizes both cell lines to cisplatin-induced cytotoxicity ($p < 0.05$, $p < 0.01$, $p < 0.001$, two-way ANOVA). (**E–F**) Wound healing assays in HeLa (**E**) and SiHa (**F**) cells transfected with si-Control or si-USP14. Knockdown of USP14 significantly impairs cell migration as measured by wound closure percentage at 24 h ($p < 0.05$, unpaired *t*-test). (**G–H**) Transwell invasion assays of HeLa (**G**) and SiHa (**H**) cells following USP14 knockdown. Quantification of invasive cells shows a mild decrease that is not statistically significant (*ns*, not significant, unpaired *t*-test). Data are represented as mean ± SEM from three independent experiments. Statistical significance is indicated as follows: $p < 0.05$, $p < 0.01$, $p < 0.001$, and ns = not significant

3.3 Effect of IU1 on cervical cancer cell proliferation and migration

Since our findings indicate that USP14 inhibition reduces CC cell proliferation and migration. To further corroborate and expand upon the siRNA-based observations, the USP14-specific inhibitor IU1, which targets its catalytic activity, was used to assess its impact on the proliferative and migratory behaviour of CC cells. Therefore, to evaluate the impact of IU1 treatment on CC cell proliferation, colony formation assays were conducted following treatment with IU1 at standardized concentrations of 50μM and 100μM. The results demonstrated a dose-dependent reduction in colony numbers, indicating that IU1 markedly inhibits the proliferative potential of both HeLa (Figure 3A) and SiHa (Figure 3B) cells.

Similarly, to investigate the effect of pharmacological inhibition of USP14 on the migration of CC cells, scratch wound healing assays were conducted following treatment with the USP14 inhibitor IU1 at concentrations of 50μM and 100μM. Following IU1 treatment, wound closure was monitored over time (0-24h). The results demonstrated a dose-dependent decrease in wound healing, with a marked reduction in wound closure observed after 24h at both concentrations as compared to control DMSO in both HeLa (Figure 3C) and SiHa (Figure 3D) Cells. Additionally, transwell migration assays were performed to validate further the role of USP14 in regulating CC cell migration following treatment with the USP14 inhibitor IU1 at concentrations of 50 μM and 100 μM. Results demonstrated a significant reduction in the number of cells migrating through the membrane in HeLa (Figure 3E) and SiHa cells (Figure 3F) upon IU1 treatment as compared to DMSO.

Collectively, these findings corroborate our earlier findings and demonstrate that pharmacological inhibition of USP14 and IU1 effectively reduces both proliferation and migration of the cervical cancer cells, supporting its potential as a therapeutic strategy.

Figure 3. Effect of IU1 on cervical cancer cell proliferation and migration. (A–B) Colony formation assays showing reduced clonogenic survival in HeLa (**A**) and SiHa (**B**) cells treated with IU1 (50 μM and 100 μM) compared to DMSO control. Quantification indicates a dose-dependent reduction in colony formation ($p < 0.05$, $p < 0.01$, $p < 0.001$, one-way

ANOVA). (**C–D**) Wound healing assays demonstrating that treatment with IU1 significantly impairs migration of HeLa (**C**) and SiHa (**D**) cells in a dose-dependent manner. Quantification of wound closure at 24 h is shown below ($p < 0.05$, $p < 0.01$, $p < 0.001$, one-way ANOVA). (**E–F**) Transwell invasion assays in HeLa (**E**) and SiHa (**F**) cells treated with DMSO or IU1 (50 μM and 100 μM). IU1 significantly reduces the number of invading cells in both cell lines compared to control ($p < 0.05$, $p < 0.01$, $p < 0.001$, one-way ANOVA). All data are presented as mean ± SEM from three independent experiments. Statistical significance is indicated as follows: $p < 0.05$, $p < 0.01$, $p < 0.001$, and ns = not significant

3.4 Proteomic Analysis to identify the molecular pathways modulated by the disruption of USP14 activity.

To understand the complex interplay of different pathways involving USP14, we used the proteomic approach to identify the molecular pathways modulated by the disruption of USP14 activity. To explore the potential interacting partners and downstream effects of USP14, a global proteomic analysis was conducted following the silencing of USP14 using siRNA in SiHa cells. Proteomic profiling was performed in both control and USP14 knockdown groups to identify differentially expressed proteins (Supplementary File 1). The results revealed significant proteomic alterations upon USP14 inhibition, illustrated by a volcano plot showing markedly upregulated (red) and downregulated (blue) proteins (Figure 4A). A detailed list ofsignificantly dysregulated proteins, highlighting the broad impact of USP14 silencing (Supplementary File 2)

To understand the biological relevance of these dysregulated proteins, Reactome pathway and GO enrichment analyses were performed. Protein lists, categorized by upregulation and downregulation based on fold change and adjusted p-values, were used with gene symbols or UniProt IDs. Furthermore, the Reactome pathway analysis and GO enrichment categorized proteins under Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), and R-based packages were used for deeper statistical analysis and visualization through dot plots and bar graphs. Reactome pathway analysis revealed that USP14 inhibition led to distinct alterations in functional networks. Upregulated pathways included RNA

polymerase II transcription termination, mRNA splicing, processing of capped intron-containing pre-mRNA.(Figure 4B). Conversely, downregulated pathways were related to neutrophil degranulation, Gluconeogenesis , MAPK signaling, and metabolism of carbohydrates indicating suppression of USP14 results in alteration in inflammatory, immune response , and metabolic regulation. (Figure 4C).

Moreover, GO enrichment analysis following USP14 knockdown in SiHa cells revealed significant alterations in molecular functions. Upregulated proteins were predominantly associated with ubiquitin-dependent processes, including ubiquitin-like protein ligase binding, ubiquitin-like modification–dependent protein binding, K63-linked polyubiquitin modification, and ionotropic glutamate receptor binding (Figure 4D). In contrast, downregulated proteins were mainly involved in phosphatidylinositol acyl-chain remodeling, regulation of triglyceride metabolism, and cell migration (Figure 4E)

Additionally, GO analysis of biological processes (Figure S3A and B) and cellular components (Figure S3C and D) further supported these observations. Upregulated proteins were mainly enriched in pathways related to the regulation of protein ubiquitination, post-translational modification, catalytic spliceosome activity, and focal adhesion, whereas downregulated proteins were associated with phosphatidylinositol acyl-chain remodeling, triglyceride metabolism, the mitochondria-associated endoplasmic reticulum membrane, and the DNA repair complex. These findings suggest that USP14 plays a pivotal role in regulating mitochondrial function, genome stability, and protein homeostasis, and its inhibition leads to widespread disruption of these critical cellular pathways. In summary, these findings highlight USP14 as a central regulator of oncogenic and metastatic signalling in cervical cancer. Its inhibition disrupts critical cellular pathways and alters the abundance of proteins involved in tumor development, emphasizing its potential as a promising therapeutic target.

Figure 4. Proteomic Analysis to identify the molecular pathways modulated by the disruption of USP14 activity. (**A**) Volcano plot displaying differentially expressed proteins in SiHa cells transfected with si-control versus si-USP14. Proteins with significant upregulation (red) and downregulation (blue) are highlighted (Threshold: $\log_2$ fold change > 1 and $p < 0.05$). (**B-C**) Reactome pathway enrichment analysis of significantly upregulated (B) and downregulated

(**C**) proteins following USP14 inhibition. Pathways are ranked by gene count and significance (p-adjusted < 0.05). (**D-E**) Gene Ontology (GO) molecular function enrichment analysis showing top upregulated (**D**) and downregulated (**E**) molecular function upon USP14 knockdown. Dot size represents gene count; color intensity reflects statistical significance (p-adjusted < 0.05)

3.5 The interaction of USP14 with SLC16A3 (MCT4) and its downstream targets identification

Proteomic analysis has revealed that USP14 is involved in the regulation of the cell cycle, stress response, and metabolic pathways. Studies suggest that USP14 regulates various factors and molecular targets at both the RNA and protein levels in different cancers, influencing pathways and biological processes such as autophagy and metabolism (25,38). Another study indicates that USP14 plays a key role in cellular metabolism by stabilizing fatty acid synthase (FASN), thereby promoting lipid accumulation and contributing to hepatosteatosis (39).

In this study, stable isotope labelling with amino acids in cell culture (SILAC)-based quantitative proteomics was conducted in HeLa cells following USP14 knockdown to systematically identify its downstream targets involved in cellular metabolism. This proteomic approach revealed significant alterations in the expression of proteins associated with lipid and carbohydrate metabolic pathways, including a notable dysregulation of the lactate transporter SLC16A3, also known as Monocarboxylate Transporter 4 (MCT4). The altered expression of MCT4 upon USP14 inhibition suggested a potential regulatory relationship between the two proteins (40).

To further investigate the expression and interaction profile of MCT4 in CC cells, USP14 was first inhibited by using siRNA. Following successful knockdown, MCT-4 expression was evaluated by immunoblotting. The results showed a marked reduction in MCT4 levels in both HeLa (Figure S4A) and SiHa (Figure 5A) cells upon USP14 knockdown, compared to the siControl group, indicating that MCT4 is likely a downstream target regulated by USP14. To further validate this functional relationship, CC cells were treated with the USP14 inhibitor IU1 at concentrations of 50 μM and 100 μM for 24 hr, and MCT4 expression was subsequently assessed. Immunoblotting was performed, and the results revealed a dose-

dependent decrease in MCT4 expression in both HeLa (Figure S4B) and SiHa (Figure 5B) cells following IU1 treatment, supporting the regulatory role of USP14 in modulating MCT-4 levels.

Additionally, to understand whether USP14 affects the subcellular localization of MCT4, immunofluorescence staining was performed in SiHa cells after USP14 knockdown. The imaging revealed a noticeable reduction in membrane-associated MCT4 in USP14-depleted cells, indicating that USP14 may influence both the expression and localization of MCT4. (Figure S4C). To further explore the potential interaction between USP14 and MCT4, co-immunoprecipitation (Co-IP) assays were conducted. Cell lysates from SiHa cells were immunoprecipitated with an anti-USP14 antibody and probed with an anti-MCT4 antibody (Figure 5C), which confirmed the endogenous interaction between USP14 and MCT4. This interaction was further validated by a reverse co-immunoprecipitation (Co-IP) using an anti-MCT4 antibody, followed by probing with an anti-USP14 antibody (Figure 5D).

As USP14 is a deubiquitinating enzyme (DUB) that removes ubiquitin chains from its substrates, we further investigated whether USP14 regulates MCT4 stability through deubiquitination. A ubiquitination assay was performed in which SiHa cells were co-transfected with a GFP-tagged ubiquitin (GFP-Ub) plasmid, along with either siControl or si-USP14. Immunoprecipitation using anti-MCT4 antibody followed by immunoblotting with an anti-GFP antibody revealed increased ubiquitination of MCT4 and its complex proteins in USP14-silenced cells, suggesting that USP14 removes ubiquitin from MCT4 and maybe other proteins associated with it to stabilize it (Figure 5E). To further validate this, a similar assay was conducted in USP14-overexpressing cells co-transfected with an HA-tagged ubiquitin (HA-Ub) plasmid and either an empty vector or His-tagged USP14. Results indicate that the Immunoprecipitation with anti-MCT4 and probing with anti-HA antibody showed an increase in DUB activity upon overexpression of USP14 (Figure 5F

Overall, these findings suggest that USP14 interacts with MCT4 endogenously and may regulate its expression and membrane localization through deubiquitination, thereby influencing its stability and function in cervical cancer cells.

Figure 5. The interaction of USP14 with SLC16A3 (MCT4) and its downstream targets identification. (**A**) Western blot analysis showing the effects of USP14 knockdown (si-USP14) on MCT4 protein levels in SiHa cells. siRNAs targeting USP14 significantly reduce both USP14 and MCT4 levels compared to the control. Actin was used as a loading control. Quantification of MCT4 levels normalized to Actin is shown on the right ($p < 0.05$, $p < 0.01$, one-way ANOVA). (**B**) Treatment with USP14 inhibitor IU1 (50 μM and 100 μM) also results in a dose-dependent reduction in MCT4 protein expression in SiHa cells. Actin serves as a loading control. Quantification is shown on the right ($p < 0.05$, $p < 0.01$, one-way ANOVA). (**C**) Co-immunoprecipitation (co-IP) assay showing that endogenous USP14 interacts with MCT4. Lysates from SiHa cells were immunoprecipitated with anti-USP14 and blotted with anti-MCT4. The red box highlights the specific MCT4 band. (**D**) Reverse co-IP confirms the interaction. Lysates were immunoprecipitated with anti-MCT4 and blotted with anti-USP14. The red box highlights the specific USP14 band. (**E**) Ubiquitination assay in GFP-Ub transfected cells shows increased MCT4 ubiquitination upon USP14 knockdown, indicating that USP14 may deubiquitinate MCT4. (**F**) In vitro ubiquitination assay using HA-tagged ubiquitin and His-tagged USP14 demonstrates that USP14 overexpression reduces MCT4 ubiquitination levels, confirming its role in MCT4 deubiquitination. Data are presented as mean ± SEM from three independent experiments. Statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**)

3.6 Molecular Docking and Structural Analysis of USP14 and MCT4 Interaction

The human MCT4 protein is a 465-amino-acid, 12-helix transmembrane protein with an extended cytoplasmic tail (Figure 6B). In contrast, the human full-length deubiquitinating enzyme USP14 consists of 494 residues and features an N-terminal Ubiquitin-like (UBL) domain (residues 1-95) along with a C-terminal catalytic domain (residues 96-494). The catalytic domain includes USP activating sub-domain BL1 (residues 329-351) and BL2 (residues 429-433) (Figure 6A). It is well established that the UBL domain of USP14 interacts with and regulates 26S proteasome activity (41)., while also exerting an allosteric inhibitory effect on proteasomal function (42). We utilized the structure of the catalytic domain of USP14. Molecular docking analysis of USP14's catalytic domain with MCT4, performed using ClusPro, indicated the formation of a stable complex (Figure 6C). The

docking results yielded a weighted score of -1894.9, with a cluster containing 101 members, highlighting a strong binding interaction. Upon analyzing the best-docked pose, 29 USP14 residues and 28 MCT4 residues were found to be involved in the interactions (Figure S5A). The USP14-MCT4 complex had 3 salt bridges, 10 H-bonds, and 161 non-bonded contacts (i.e., hydrophobic or van der Waals interactions) within the two proteins (Figure S5B).

To further assess the stability of the USP14-MCT4 complex, molecular dynamics (MD) simulations were conducted for 200 ns (Figure 6C). The RMSD remained stable throughout the MD simulations, while a decrease in Rg and SASA indicated enhanced binding stability (Figure S6A-S6C). Apart from the cytoplasmic tail of the USP14, the rest of the protein exhibited stable RMSF, signifying structural consistency in the loop regions (Figure S6D). Superimposition of 0 and 200 ns frames revealed that the complex remained structurally stable, with no major conformational change. In contrast, USP14 exhibited minimal residue-wise fluctuations, confirming the overall structural rigidity and a stable interaction with MCT4 (Figure S6E). Superimposition of 0 and 200 ns frames further demonstrated that the complex remained structurally stable, with no major conformational change (Figure S6D).

To examine the interacting interface of USP14-MCT4, contact mapping was performed for both 0 ns and 200 ns frames (Figure S7A and Figure S7B). Our results showed that even after 200 ns, the interacting interface remained largely unchanged. Additionally, Structural evaluation of both frames identified 10 interacting residue pairs that persisted throughout the simulations, further identified 10 interacting residue pairs that persisted throughout the simulations, further confirming the formation of a stable protein complex. (Figure S5B and Figure S5D) . At 200ns of MD simulations, 1 salt bridge, 9 hydrogen bonds and 114 non-bonded contacts were still present, further emphasizing the robust stability of the USP14-MCT4 complex (Figure S5C and S5D).

Figure 6. Molecular docking and structural analysis of USP14 and MCT4 interaction. (**A**) Schematic representation of the USP14 catalytic domain highlighting its structural features. The lower panel displays the 3D structure of the USP14 catalytic domain (residues 96-494), with key active site residues, C114 (nucleophile) and H435 (proton acceptor), highlighted.

(**B**) Structural model of MCT4 (SLC16A3) highlighting transmembrane (TM) helices (colored H1–H12) and the cytoplasmic domain. Predicted ubiquitination sites (K45, K63, K81, K353, and K484) are labeled. (**C**) Molecular dynamics (MD) simulations showing structural conformations of MCT4 and USP14 at 0 ns and 200 ns. Both front and top views are presented to depict the conformational rearrangement and docking orientation over time. (**D**) Molecular docking model depicting the interaction between MCT4 and USP14. The zoomed-in view illustrates spatial proximity between MCT4 lysine residues (notably K45, K81, and K353) and the catalytic USP domain of USP14, indicating potential docking and deubiquitination sites.

3.7 Effect of USP14 Depletion on MCT4 Expression and Lactate Efflux

All the experiments conducted so far suggest that USP14 plays a role in regulating MCT4 expression, interacts with MCT4, and may influence its protein stability and degradation. To further examine whether USP14 affects the stability of MCT4, a cycloheximide (CHX) chase assay was performed to determine the half-life of MCT4 protein. In this experiment, SiHa cells were transfected with siControl and si-USP14 for 48 h. After transfection, cells from both groups were treated with 100 μg/mL CHX to inhibit new protein synthesis, and samples were collected at different time points. Western blot analysis was used to monitor MCT4 protein levels over time. Results indicate that in the siControl group, MCT4 protein levels remained relatively stable throughout the 8h period, suggesting normal protein stability (Figure 7A). In contrast, siUSP14-transfected cells exhibited a marked and progressive reduction in MCT4 levels over time, indicating that the absence of USP14 resulted in the faster degradation of MCT4 (Figure 7B). These findings suggest that USP14 contributes to the stabilization of MCT4, possibly by protecting it from degradation.

To further investigate the functional impact of USP14 on MCT4 activity, we measured extracellular lactate levels in the culture supernatants by lactic acid estimation assay following both siRNA-mediated and pharmacological inhibition of USP14. Initially, USP14 was silenced using siUSP14 in HeLa and SiHa CC cells. After 48h of transfection, the culture media from all conditions were collected, and extracellular lactate levels were quantified. The

results showed no significant change in lactate secretion between siControl and siUSP14-treated groups in HeLa cells (Figure 7C). However, in SiHa cells, USP14 knockdown led to a notable reduction in extracellular lactate levels compared to the siControl group (Figure 7D). This suggests that USP14 contributes to MCT4-mediated lactate efflux in a cell line dependent manner, likely by stabilizing the MCT4 protein. To further validate these findings, we examined the effect of pharmacological inhibition of USP14 using IU1, a specific inhibitor of its catalytic activity. Both HeLa and SiHa cells were treated with IU1 at concentrations of 50μM and 100μM. Following treatment, the supernatants were collected, and lactate concentrations were measured. The data revealed a significant decrease in extracellular lactate levels upon IU1 treatment in both HeLa (Figure 7E) and SiHa (Figure 7F) cells, supporting the idea that USP14 activity is crucial for MCT4 function.

Together, these findings indicate that USP14 supports lactate export in CC cells by stabilizing MCT4. Inhibition of USP14, either by siRNA-mediated knockdown or by pharmacological inhibition, leads to impaired MCT4 function and reduced lactate secretion. This highlights a potential regulatory mechanism of USP14 on MCT4 that may contribute to the metabolic phenotype of CC cells.

Figure 7. Effect of USP14 Depletion on MCT4 Expression and Lactate Efflux. (**A, B**) Cycloheximide (CHX) chase assay demonstrating the protein stability of MCT4 in HeLa cells transfected with si-Control (**A**) or si-USP14 (**B**) over time (0–8 h). Western blotting shows that MCT4 levels decline more rapidly upon USP14 knockdown, suggesting USP14 stabilizes MCT4. Actin is used as a loading control. (**C**) Measurement of extracellular lactate levels in HeLa cells after USP14 knockdown shows no significant change compared to control (ns, not significant; unpaired Student's t-test). (**D**) In SiHa cells, USP14 knockdown significantly reduces extracellular lactate levels compared to control ($p < 0.01$; unpaired Student's t-test). (**E**) Treatment of HeLa cells with USP14 inhibitor IU1 (50 μM and 100 μM) leads to a dose-dependent decrease in extracellular lactate concentration compared to DMSO control ($p < 0.01$, $p < 0.05$; one-way ANOVA). (**F**) IU1 treatment in SiHa cells also leads to a significant, dose-dependent decrease in lactate efflux compared to DMSO control ($p < 0.05$; one-way

ANOVA). Data are shown as mean ± SEM from three independent experiments. Statistical significance is denoted as: ns, not significant; $p < 0.05$ (*), $p < 0.01$ (**).

3.8 Analysis of altered expression of USP14 and MCT-4 in cervical cancer tissue sections

To further explore the expression of USP14 and MCT4 (SLC16A3) and their correlation in CC tissue sections as compared to normal ones, we analyzed publicly available transcriptomic data from the GSE113942 dataset, which includes seven normal and seven tumor tissue samples. The results showed elevated mRNA levels of both USP14 (Figure S8A) and MCT4 (Figure S8B) in tumor samples compared to normal tissues, with a positive correlation between their expressions. To understand the broader molecular impact of USP14, we performed differential gene expression analysis comparing tumors with high and low USP14 expression. The resulting volcano plot highlighted distinct genes significantly upregulated or downregulated, based on which are associated with the USP14 expression (Figure S9A). Notably, MCT4, which is involved in cancer metabolism, was also upregulated in USP14-high samples, indicating a functional link between USP14 and metabolic regulation. Additionally, to evaluate the impact of dysregulated proteins in both groups, a pathway enrichment analysis using Reactome and GO was done using R software and packages, and the results revealed that high USP14 expression is associated with altered metabolic pathways such as pigment metabolism, retinoid metabolism, glucuronidation, and uronic acid metabolism. (Figure S9 B and C).

Moreover, to validate these findings at the protein level and assess their clinical relevance, we performed immunohistochemistry on 30 retrospective CC tissues, mainly of primary squamous cell carcinoma, and 6 normal cervix tissues. Positive controls were included, stomach tissue for USP14 and placenta for MCT4, respectively. The positive controls showed the localization pattern of both i.e, cytoplasmic for USP14 and membranous for MCT4 (Figure S10). For scoring of the tissues for both protein expression in the samples, two parameters were considered for the final score. One is the percentage of positive cells (scale 1–4) and the second is staining intensity (scale 1–3), with the final score calculated by multiplying these values. The results demonstrated significantly higher expression of USP14

in the CC tissues as compared to the normal one (Figure 8A). Moreover, the expression of MCT4 is also found to be upregulated in CC tissue sections, but not significantly (Figure 8B). To identify the correlation between the expression of USP14 and MCT4, the Pearson correlation coefficient was further estimated using R, and its value of 0.38 confirmed a positive correlation between USP14 and MCT4 protein levels in CC tissues (Figure 8C). Collectively, these findings suggest that USP14 and MCT4 are overexpressed in CC, and that USP14 may promote tumor progression by regulating key metabolic pathways.

Figure 8. Analysis of altered expression of USP14 and MCT-4 in cervical cancer tissue sections. (**A**) Representative immunohistochemical images showing USP14 expression in cervical cancer tissues (n = 30) compared to normal tissues (n = 6), along with a corresponding box plot illustrating the differential expression levels. Representative images are shown at a scale of 124.5 μm within the square box, with the corresponding zoomed-in views displayed adjacently within the circular insets. The red arrows indicate the specific localization within the tissue USP14 shows cytoplasmic expression, while MCT4 exhibits membranous localization. (**B**) Representative images depicting MCT-4 expression in cervical cancer tissues (n = 30) versus normal tissues (n = 6), accompanied by a box plot comparing expression levels across the two groups. (**C**) Scatter plot illustrating the correlation between USP14 and MCT4 expression levels. Each dot represents an individual sample, with USP14 expression on the x-axis and MCT4 on the y-axis. The red regression line indicates a moderate positive correlation (Pearson correlation coefficient = 0.38).

4. Discussion

Resistance and recurrence in CC remain major challenges, highlighting theneed for novel molecular targets. (43). The Ubiquitin-proteasome system (UPS), a key regulator of protein stability and celluar homeostasis, has emerged as a promising therapeutic approach.

Following the success of proteasome inhibitors in haematological malignancies, attention has shifted towards the upstream components of UPS, particularly deubiquitinating enzymes (DUBs).(44). Among them deubiquitinating ubiquitin-specific proteases (USPs) have emerged as key regulators of cell cycle progression, DNA repair, chromatin remodelling, and oncogenic signalling (7,8). Dysregulated expression of various USPs has been observed in multiple cancers, where they stabilize target substrates and regulate key molecular mechanisms of cancer cells, acting as oncogenic drivers and making them attractive targets for therapeutic intervention(8).

Previous studies from our group have demonstrated that the role of USPs, particularly USP37, extends beyond its canonical role in S-phase entry, preserving replication fidelity and genome integrity through CHK1 stabilization (14). Another original study from our group indicates that USP37 depletion increased DNA damage and replication stress sensitivity, underlining its potential as a therapeutic target in osteosarcoma by stabilizing PCNA (Proliferating cell nuclear antigen), which is a master clamp loader at the replication fork (12). While exploring the role of USP37 in cancer progression, our group interestingly identified another deubiquitinating enzyme, USP14, showing altered expression across multiple cancers.

Recent studies have reported that USP14 is frequently upregulated and contributes to tumor progression through various mechanisms. Elevated USP14 stabilizes JNK and activates the MAPK/JNK signaling pathway, thereby promoting carcinogenesis and positioning it as a potential therapeutic target.(45) Moreover, USP14 serves as a biomarker for recurrence risk in endometrial cancer, co-expressing with Ki67, and its inhibition by the FDA-approved compound VLX1570 reduces chemoresistant cell viability via cell cycle arrest and apoptosis.(46) Similarly, USP14 overexpression in breast cancer correlates with higher histological grade, lymph node metastasis, and poor prognosis. (47) Therefore, these findings highlight USP14 as a critical USP family member with oncogenic potential, suggesting further investigation in cervical and other solid tumors.

As the role of USP14 in cervical cancer remains unexplored, its expression was evaluated in cervical cancer cell lines and tissues compared with normal counterparts using in-silico datasets (GEPIA2, TCGA, GTEx, and CCLE). The analysis revealed a significant overexpression of USP14 in cervical cancer, which correlated with advanced disease stage and poor patient prognosis. To strengthen the comparative analysis, USP14 expression was

also evaluated in the non-transformed epithelial cell line MCF10A, a widely accepted control model for studying transformation. The comparison revealed markedly higher USP14 expression in cervical cancer cell lines, suggesting its potential involvement in cellular transformation and tumor progression.(48,49,50)

Literature evidence indicates that USP14 regulates key cancer-associated processes such as proliferation, migration, and drug resistance, promoting tumor progression by modulating survival and metastatic pathways across multiple malignancies, including breast cancer, multiple myeloma, and hepatocellular carcinoma. (17, 19, 51) To assess its role in cervical cancer (CC), modulation of USP14 expression and inhibition with IU1 revealed that USP14 promotes CC cell proliferation, migration, and survival, while its depletion or inhibition suppresses these functions and enhances cisplatin sensitivity, underscoring its potential as a therapeutic target.

Despite accumulating functional evidence, the global downstream effectors of USP14 inhibition and its interacting oncogenic partners remain an active research area. To address this gap, we employed global proteomic profiling in CC SiHa cells following USP14 silencing. Proteomic analysis revealed that USP14 inhibition modulates multiple pathways, including ubiquitin-dependent processes, MAPK signaling, carbohydrate and triglyceride metabolism, vesicle transport, and neutrophil degranulation, while also influencing immune responses migration and metabolism, suggetsing its multifaceted role in regulating oncogenic mechanisms. The data emerging from our proteomic profiling encouraged us to further investigate the role of USP14 in metabolic reprogramming, a hallmark of cancer and leads to a notable finding that is the association between USP14 and Monocarboxylate Transporter 4 (MCT4), encoded by the SLC16A3 gene. MCT4 facilitates lactate export, aiding in intracellular pH regulation and supporting the Warburg effect. As our proteomics data suggest that there is dysregulation of MCT4 after USP14 inhibition, subsequent experiments confirmed that both siRNA-mediated knockdown and USP14 inhibitor IU1 treatment reduced MCT4 protein levels. Interestingly, the Immunofluorescence assay further revealed an altered subcellular localization of MCT4 upon USP14 inhibition Subsequently, through immunoprecipitation experiments, a direct interaction between USP14 and MCT4,was also confirmed alongwith reciprocal co-immunoprecipitation assays, implying that USP14 physically interacts with MCT4. To further determine whether USP14, as a deubiquitinase,

regulates MCT4 stability, a deubiquitination assay was performed, which revealed that USP14 removes ubiquitin chains from MCT4, thereby preventing its degradation and enhancing its stability. Moreover Cycloheximide chase assays supported this finding, revealing faster degradation of MCT4 in USP14-depleted cells altogether suggested that USP14 directly interacts with and deubiquitinates MCT4, preventing its degradation and thereby stabilizing it

Since MCT4 is a proton-linked symporter that facilitates the bidirectional transport of monocarboxylates, mainly lactate, across the plasma membrane and helps the cells to remove excess lactic acid produced during intense glycolysis or with respect to cancer under hypoxic conditions, or the Warburg effect observed in tumors. So in this study, lactate assays revealed that USP14 regulates lactate export, as both siRNA-mediated knockdown and IU1 inhibition significantly reduced extracellular lactate levels in cervical cancer cells. These observations suggest that USP14 contributes to metabolic rewiring in CC via stabilization of MCT4, supporting lactate efflux and potentially aiding uncontrolled proliferation, survival under stress, metastasis, and therapy resistance.

In order to deeply understand the molecular interaction between USP14 and MCT4, Molecular Dynamics studies followed by molecular docking suggested a a docked pose where the active site of USP14 catalytic domain-comprising Lys448, Lys453, and the BL1 & BL2 subdomains-interacts with the cytosolic domain of MCT4, which contains five ubiquitination sites (K415, K428, K431, K4553, K488). This interaction suggests the possibility that USP14 may deubiquitinate these lysine residues, thereby enhancing the half-life and stability of MCT4.

Clinically, our findings are substantiated by transcriptomic and immunohistochemical analysis. Data from the GSE113942 dataset demonstrated the upregulation of both USP14 and MCT4 in CC tissues, with positive correlation at the transcript level. Differential gene expression analysis between USP14-high and USP14-low tumors further revealed the upregulation of genes involved in cancer metabolism, including pigment and retinoid metabolism as well as glucuronidation pathways. Moreover,USP14 and MCT4 expression analysis in archived CC patient tissue samples confirmed overexpression of USP14 and MCT4, with Pearson correlation analysis ($r = 0.38$) affirming a moderate positive association. The cytoplasmic localization of USP14 and the membranous distribution of MCT4 were consistent with their known subcellular functions. Our MD simulation revealed that the

catalytic domain of USP14 can stably bind and stabilize the transmembrane region of MCT4. Given that MCT4 is synthesized in the endoplasmic reticulum (ER) and is transported to the plasma membrane via vesicular transport, its stability during this process is crucial. According to the Human Protein Atlas (https://www.proteinatlas.org/), USP14 is localized to the plasma membrane, cytosol, and ER. This suggests that USP14 could potentially enter the ER, where it may contribute to MCT4 stabilization before its transport to the membrane. USP14 might influence MCT4 folding or stability within the ER. It could play a chaperone-like role in ensuring efficient maturation and trafficking of the protein (Figure 9).

Figure 9. Illustrating the role of USP14 in regulating MCT4-mediated lactate export and EMT activation in cancer progression. In normal conditions, USP14 stabilizes MCT4, leading to increased lactate efflux, activation of the epithelial-mesenchymal transition (EMT) pathway, and promotion of cancer progression/metastasis. Inhibition of USP14, either pharmacologically with IU1 or by siRNA-mediated knockdown, reduces MCT4 stability and lactate export, thereby suppressing EMT activation and inhibiting cancer progression/metastasis.

Based on these findings, we propose a dual role for USP14 in regulating MCT4 stability and function. Firstly, USP14 may enter the ER, where it assists in MCT4 folding and imparts stability during its maturation, thereby enhancing MCT4 turnover. Additionally, USP14 may perform its deubiquitinating function, removing ubiquitin modifications from five predicted lysine residues (K415, K428, K431, K4553, K488), which could contribute to MCT4 stability by extending its half-life and supporting its functional regulation. Taken together, our findings underscore USP14 as a multifaceted oncogenic regulator in CC and identify MCT4 as a novel interacting partner. Our proteomic analysis on USP14-depleted cells indicates its functional roles spanning proliferation, migration, metabolic reprogramming, and chemoresistance. Mechanistically, USP14 appears to promote these processes partly through stabilization of MCT4, linking it directly to the metabolic phenotype of CC cells. These insights underscore its utility as a promising target in CC. Available USP14 inhibitors such as

IU1, b-AP15, and WP1130 have shown preclinical efficacy in multiple cancers, but their optimization for specificity and *in vivo* potency remains an active area of research. Notably, USP inhibitors targeting other family members, such as P5091 (USP7), ML364 (USP2), and spautin-1 (USP10/13), further highlight the growing interest in this enzyme class as druggable targets. Future research should focus on identifying additional USP14 substrates and cofactors involved in oncogenesis and drug resistance in cancer-specific studies. High-throughput screening, interactome mapping, and CRISPR-based functional genomics can accelerate the discovery of novel therapeutic targets within this pathway. Moreover, integrating USP14 inhibition with existing treatment modalities, such as chemotherapy or immune checkpoint blockade, may yield synergistic effects and improve patient outcomes in CC. Ultimately, *in vivo* validation using orthotopic or patient-derived xenograft models, alongside pharmacokinetic and pharmacodynamic studies, will be essential to advance USP14-targeted therapies from the bench to the bedside.

Conclusion: Our study provides compelling evidence for the oncogenic role of USP14 in CC and identifies MCT4 as a target of USP14 in CC, particularly through its regulation of metabolic reprogramming and drug resistance. By stabilizing MCT4 and modulating multiple oncogenic pathways, USP14 emerges as a promising prognostic biomarker and therapeutic target. Pharmacological inhibition of USP14, alone or in combination with standard therapies, represents a novel and rational approach to improve CC management.

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Author contributions

R.C., G.D, L.M., A.G., H.K., H.Y., R.U., S.K.D., and M.S. conducted experiments and wrote the manuscript. M.S. and A.A.B. contributed to the concept, design, writing and critically edited the manuscript. M.S., M.A.M., S.M., A.S, M.T., P.T., E.A.S., A.S, A.S.A.A., S.U., and T.K.P. helped in the interpretation of Data and performed critical revision and editing of the scientific content. All authors read and approved the final manuscript.

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Data availability

The authors declared that all data supporting the conclusions of this research are available in the main manuscript and the supplementary files.

Declarations

Ethics approval and consent to participate

Ethical approval for the current study was obtained from AIIMS Institute ethics committee (AIIMS IEC) bearing no AIIMSA1578/27.06.24, RT-16/25.07.2024

Consent for publication

All authors consent to publication.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

AC Adenocarcinoma
AIS Adenocarcinoma in situ
ASC Adenosquamous carcinoma
ASR Age-standardized rate
ATP Adenosine triphosphate
CCLE Cancer Cell Line Encyclopedia
CESC Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHX Cycloheximide
CIN Cervical intraepithelial neoplasia
DFS Disease-free survival
DMSO Dimethyl sulfoxide
DUBs Deubiquitinating enzymes
EBRT External beam radiation therapy
E6AP E6-associated protein
EMT Epithelial-mesenchymal transition
FASN Fatty acid synthase
FDA Food and Drug Administration
FIGO International Federation of Gynecology and Obstetrics
GEPIA2 Gene Expression Profiling Interactive Analysis 2
GO Gene Ontology
GTEx Genotype-Tissue Expression
HPV Human papillomavirus
HSIL High-grade squamous intraepithelial lesion
IHC Immunohistochemistry
IQR Interquartile range
LBC Liquid-based cytology
LSIL Low-grade squamous intraepithelial lesion
MAPK Mitogen-activated protein kinase
MCT4 Monocarboxylate transporter 4
MDM2 Mouse double minute 2 homolog
MRI Magnetic resonance imaging

MSI-H Microsatellite instability–high

MRNA Messenger RNA

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

OS Overall survival

Pap Papanicolaou

PCNA Proliferating cell nuclear antigen

PD-L1 Programmed death ligand 1

PET/CT Positron emission tomography/computed tomography

pRb Retinoblastoma protein

RNA Ribonucleic acid

SCC Squamous cell carcinoma

siRNA Small interfering RNA

TCGA The Cancer Genome Atlas

UPS Ubiquitin–proteasome system

USPs Ubiquitin-specific proteases

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