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'Nelfinavir sensitizes a clinically relevant chemo-radioresistant cervical cancer *in-vitro* model by targeting the AKT-USP15/USP11-HPV16 E6/E7 axis

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ABSTRACT

Resistance to standard therapies is a major challenge in managing cervical cancer, often leading to systemic relapse. This study aimed to develop an *in-vitro* model of chemo-radioresistant cervical cancer that mimics clinical conditions and also explore the therapeutic potential of the repurposed drug nelfinavir, an HIV protease inhibitor. HPV16-positive SiHa cervical cancer cells were subjected to concurrent cisplatin and ionizing radiation, to simulate the clinical treatment regimen for locally advanced cervical cancer. The resulting chemo-radioresistant subline exhibited increased IC_{50}-value, D0 dose, and a higher Resistance Index compared to parent cells, indicating resistance development. Notably, elevated HPV16 E6/E7 expression in resistant sublines suggested a role for HPV16 in resistance acquisition. Treatment with nelfinavir significantly reduced the IC_{50}-value and D0 dose in resistant cells. Additionally, exposure to nelfinavir or AKT inhibitor IV showed significant decrease in AKT, USP15, USP11 and HPV16 E6/E7 proteins. Furthermore, siRNA mediated knockdown of USP15 and USP11 in resistant cells resulted in significant reduction of HPV16 E6 and E7 oncoproteins respectively. Thus, mechanistically nelfinavir sensitized resistant cervical cancer cells by inhibiting the AKT-USP15/USP11-HPV16 E6/E7 pathway. Overall, this study successfully established a chemo-radioresistant SiHa cell model, providing a platform for investigating resistance mechanisms. It also highlights nelfinavir's potential as a therapeutic agent in overcoming chemo-radioresistance in cervical cancer.

1. Introduction

Worldwide, cervical cancer ranks fourth among female malignancy with an estimated 604,127 new cases and 341,831 deaths annually [1]. Approximately 60 % of patients are diagnosed with locally advanced cervical cancer (LACC), leading to high morbidity and mortality rates [2]. Persistent infection with high-risk human papillomaviruses (HR-HPVs), particularly HPV16, accounts for 70 % of cervical cancer cases globally, including India [3]. The oncogenic potential of HPV is linked to integration of its DNA into the host genome. The deregulated expression of HPV oncoproteins E6 and E7, drive cellular transformation by degrading key cell cycle regulators, p53 and Rb proteins, respectively [3].

Cisplatin-based concurrent chemoradiotherapy (CCRT) followed by brachytherapy is the standard treatment for LACC, resulting in a 5-year disease-free and overall survival rate of 50–55 % and 54 % respectively [4]. However, >30 % of patients experience distant relapse within the first two years after treatment [5]. The poor clinical response is primarily due to acquired therapy resistance. Thus, highlighting the critical need to detect therapy resistance, enabling clinicians to tailor alternative treatment strategies.

Moreover, high-throughput studies have identified biomarker of chemo-radioresistance using cervical tumor tissues, however the underlying molecular mechanisms remain poorly understood [6,7]. Numerous *in-vitro* models of either chemoresistance or radioresistance have been developed to study resistance mechanisms [8,9], but there is a

Abbreviations: HR-HPV, High risk Human papilloma virus; CCRT, Concurrent chemo-radiotherapy; LACC, Locally advanced cervical cancer; IR, Ionizing radiation; RI, Resistance index.

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lack of an *in-vitro* model that replicates the clinical CCRT regimen administered to LACC patients. Such a model could provide valuable insights into the molecular events arising during development of chemo-radioresistance.

To overcome chemo-radioresistance and improve clinical outcomes in LACC, several novel therapeutic strategies have been explored, including hyperthermic radiosensitization [10], targeted immunotherapy [11], and drug repurposing [12]. Although targeted immunotherapies have shown improved clinical outcomes [11], challenges related to accessibility and affordability hinder their widespread clinical implementation. Additionally, current HPV vaccines are prophylactic, while therapeutic vaccines targeting HPV16/18 E6/E7 proteins, such as ADXS11-001, INO-3112, HPV16-SLP, and TA-CIN + GPI-0100, are in clinical trials for advanced cervical cancer [13]. So far, no therapeutic drugs targeting HPV oncoproteins exist. Alternatively, drug repurposing presents a promising approach due to its lower cost, shorter development timelines, and existing pharmacological data. Notable examples include, thalidomide for multiple myeloma, sunitinib for gastrointestinal stromal tumors, and tamoxifen for metastatic breast cancer [14]. The Repurposing Drugs in Oncology (ReDO) database has identified nelfinavir, as a potential candidate for cervical cancer treatment [12]. Nelfinavir is a FDA approved HIV protease inhibitor, that blocks HIV-1 protease, leading to immature, and non-infectious viral particles. Nelfinavir now being replaced in HIV therapy by second generation protease inhibitors. It has shown interesting *in-vitro* and *in-vivo* off-target anti-cancer effects, such as endoplasmic reticulum stress-unfolded protein response, proteasome pathway, autophagy, apoptosis, oxidative stress in different cancers including cervix [15]. However, nelfinavir's response to chemo-radioresistance remains unexplored.

Thus, in the present study, we aimed to establish an *in-vitro* chemo-radioresistant HPV16-positive cervical cancer model that simulates the clinical CCRT regimen. We comprehensively evaluated this model by analysing its drug response through IC_{50}-value determination, assessing radiation response via clonogenic assays at varying doses, and examining HPV status by quantifying HPV16 E6/E7 DNA, mRNA, and protein levels. This multi-faceted characterization aimed to provide deeper insights into the biological mechanisms underlying chemo-radioresistance. Furthermore, we explored therapeutic potential of repurposed drug nelfinavir in overcoming chemo-radioresistance in this model.

2. Materials and methods

2.1. Cell line and culture conditions

The HPV16-positive cervical cancer cell line SiHa [16] was procured from NCCS-Cell Repository, India. Authentication of cell line was confirmed through short tandem repeat profiling, using 16 markers at DNA Labs, India, with identity verified through DSMZ database [17]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), supplemented with 10 % Fetal Bovine Serum (FBS) (HiMedia, India), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (HiMedia). Cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere, with periodic mycoplasma screening to ensure cell line integrity.

2.2. Drugs and radiation treatment

Cisplatin (F.K. Oncology Ltd., India), nelfinavir mesylate (Selleckchem, USA), and AKT inhibitor IV (Sigma, USA) were used according to manufacturers' instructions. Radiation exposure was carried out at a dose rate of 300 cGy/min using 6 MV Linear accelerator (Varian, USA).

2.3. Establishment of chemo-radioresistant cell line model

To establish chemo-radioresistant cervical cancer *in-vitro* model, the HPV16-positive SiHa cell line was selected due to high prevalence of HPV16 in cervical cancer cases [3]. Chemo-radioresistant sublines were derived from parental SiHa-P cells by mimicking clinical treatment regimen. Briefly, patients receive cisplatin once weekly (40 mg/m^2) for five cycles, along with fractionated radiotherapy at 2 Gy per day, with a cumulative dose of 46 Gy in 23 fractions (Fig. 1A).

2.4. MTT assay

Both parental and chemo-radioresistant sublines were plated in triplicates in 96-well plate at a density of 5×10^3 cells/well. Cells were exposed to following treatments.

1. A gradient of cisplatin concentrations (5–60 μM) alone or in combination with 2 Gy ionizing radiation (IR)
2. Nelfinavir (20 μM) for 24 h, followed by increasing concentrations of cisplatin.

After 72 h of incubation at 37 °C, MTT (5 mg/mL) was added and incubated for 4 h, followed by overnight incubation with acidified SDS at 37 °C. Absorbance at 540 nm and 690 nm was measured to determine cell survival. Mean IC_{50}-value ± SD were calculated using GraphPad prism V9 software. Resistance index (RI) was determined using the formula:

$$\text{Resistance Index (RI)} = \text{Mean } IC_{50} \text{ of resistant subline} \,/\, \text{Mean } IC_{50} \text{ of parent cells}$$

2.5. Clonogenic assay

Clonogenic assays were performed on both parent and chemo-radioresistant sublines as previously described [18].

2.6. Western blotting

Cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.5 % SDS) supplemented with protease and phosphatase inhibitors. Protein concentrations estimated using Folin-Lowry method, followed by western blotting as described previously [18]. Antibodies used in present study are tabulated in Table S1.

2.7. Quantitative real-time PCR

DNA and RNA extractions were performed using GeneJET DNA/RNA Purification Kit (Thermo Scientific, USA). Quantitative real-time PCR for HPV16 DNA was conducted as per established protocols [19]. For mRNA quantification, total RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, USA). Expression of HPV16 E6 and E7 genes were quantified using PowerUp SYBR Green Master Mix (Applied Biosystems) on the ABI QuantStudio 12 K Flex System. GAPDH served as internal control, and primer sequences used are tabulated in Table S2.

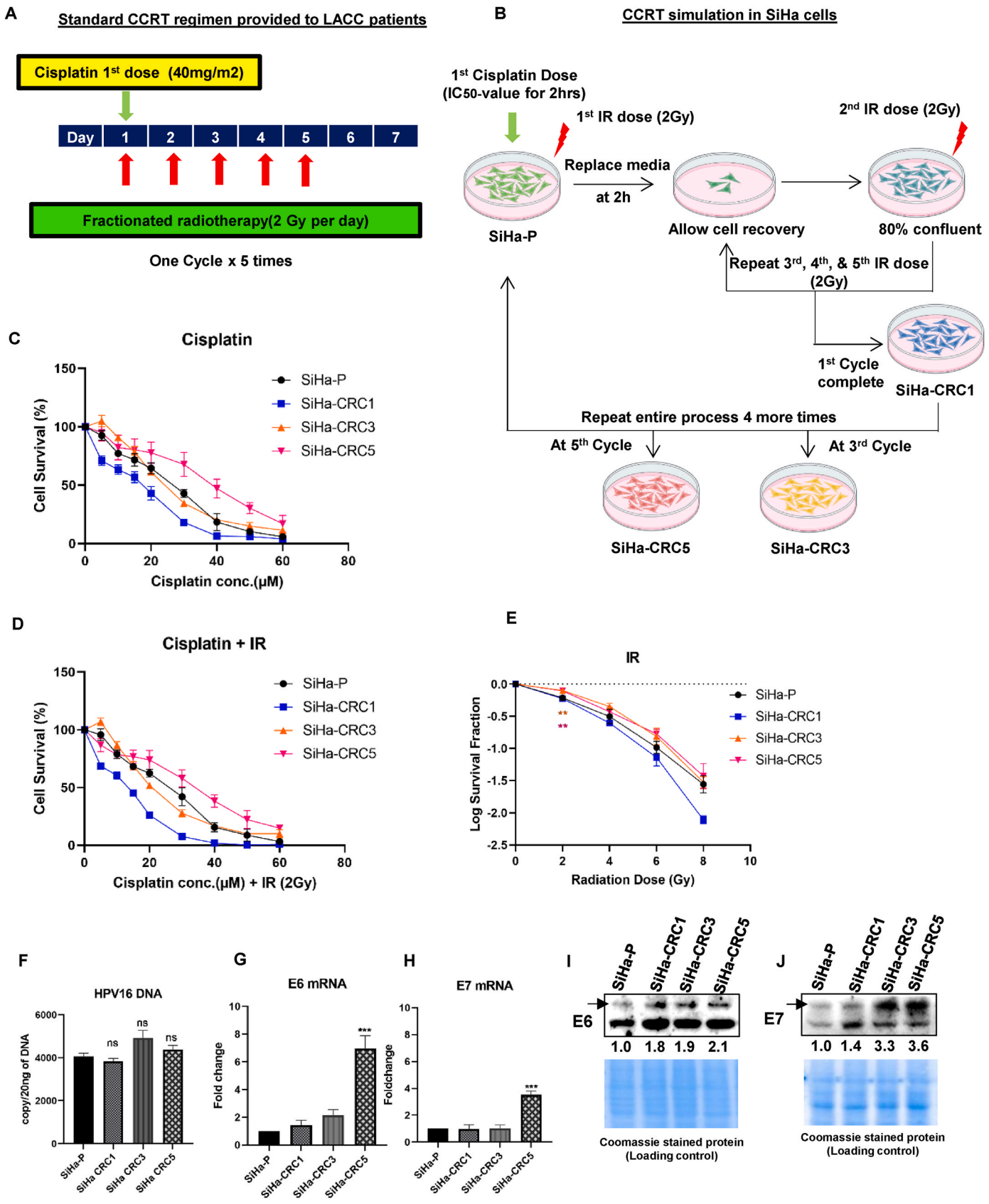

Fig. 1. Establishment and characterization of chemo-radioresistant model: (A) Schematic representation of clinical CCRT regimen **(B)** *In-vitro* simulation of CCRT to develop chemo-radioresistant SiHa sublines. **(C&D)** IC_{50}-values of cisplatin were evaluated in parental and chemo-radioresistant sublines by measuring cell survival after cisplatin alone or combined with 2 -Gy IR, using MTT assay. **(E)** Clonogenic assay comparing log survival fraction at increasing doses of IR between parental and chemo-radioresistant sublines. **(F)** Quantitative estimation of HPV16 DNA copy number in sublines using RT-PCR. Evaluation of HPV16 E6/E7 mRNA **(G&H)** and protein expression **(I&J)** in sublines using RT-PCR and Western blot, respectively.

2.8. siRNA transfection

A pool of four different siRNA oligonucleotides (ON-TARGET SMARTpool) against USP15 (Cat #: L-006066-00-0005) and USP11 (Cat #: L-006063-00-0005), were procured from Dharmacon, USA. siRNA transfection in parent and chemo-radioresistant subline was performed as described previously [18].

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism V9. Data are presented as means ± SD, based on at least three independent experiments performed in triplicates. Statistical differences between parental and resistant cell lines were assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Statistical significance: *P < 0.05.

3. Results

3.1. Development of a chemo-radioresistant HPV16-positive cervical cancer in-vitro model

To establish chemo-radioresistant cervical cancer model, SiHa-P cells (HPV16 positive cervical cancer parental line) were cultured to 80 % confluence at 37 °C with 5 % CO_2. Cells were treated with cisplatin at a concentration equivalent to the IC_{50}-value 22.31 ± 1.87 μM (derived post treatment of SiHa-P cells with gradient concentrations of cisplatin 5–60 μM along with 2Gy irradiation using MTT assay). Following cisplatin treatment, cells were exposed to a dose of 2 Gy of irradiation. This mimics the first dual dose of cisplatin with irradiation of 1st CCRT cycle. After 2 h of incubation at 37 °C with 5 % CO_2, cells were supplemented with drug-free media and allowed to recover to ensure survival of the most resistant population. The surviving cells, at 80 % confluency were then subjected to a second dose of only 2 Gy of irradiation and returned to standard culture conditions for recovery. Similarly, three additional doses of only 2 Gy of irradiation were repeated to complete 1st treatment cycle. This entire regimen of CCRT was repeated for a total of five cycles (Cisplatin once/cycle) and 46 Gy of total irradiation in 23 fractions to ensure progressive selection of chemo-radioresistant cell populations. The cells obtained on completion of 1st, 3rd, and 5th CCRT cycles were preserved and designated as SiHa-CRC1, SiHa-CRC3, and SiHa-CRC5 sublines, respectively (Fig. 1B). The development of chemo-radioresistance in these sublines was evaluated using MTT and clonogenic assays. This stepwise and rigorous selection protocol mimics clinical chemo-radiotherapy regimens.

3.2. Assessment of acquired chemo-radioresistance

MTT assay was used to assess cell survival of both parent and chemo-radioresistant sublines upon exposure to increasing concentrations of cisplatin (5–60 μM), alone and in combination with 2 Gy of irradiation. Dose-response curves are illustrated in Fig. 1C and D. Among the sublines, SiHa-CRC5 exhibited highest resistance index of 1.52- and 1.32-fold on exposure to cisplatin alone and in combination with 2 Gy of irradiation respectively as compared to SiHa-P cells. Clonogenic assay further confirmed increased survival of SiHa-CRC3 and SiHa-CRC5 sublines at 2 Gy (Fig. 1E) as compared to SiHa-P cells, indicating that these sublines had acquired enhanced resistance to chemo-radiotherapy. The IC_{50}-values of cisplatin and D0 dose for all the sublines and parent cells are tabulated in Table 1.

3.3. Upregulated HPV16 E6/E7 expression in chemo-radioresistant sublines

Previous studies from our lab have shown that persistence of high-risk HPV DNA in cervical cancer is linked to early relapse in LACC patients [19]. To explore potential role of HPV16 in acquired resistance, we quantified levels of HPV16 E6 and E7 DNA, mRNA, and protein in both parent and resistant sublines. While no significant change in HPV16 DNA copy number was observed between groups (Fig. 1F), SiHa-CRC5 demonstrated a 7-fold increase in E6 mRNA and a 3.5-fold increase in E7 mRNA in comparison to SiHa-P E6 and E7 mRNA respectively (Fig. 1G and H). Additionally, a marked upregulation of E6 and E7 proteins was observed in SiHa-CRC5 cells compared to SiHa-P cells (Fig. 1I and J), suggesting a possible role of HPV16 E6/E7 expression and acquirement of chemo-radioresistance.

3.4. Nelfinavir sensitizes chemo-radioresistant SiHa-CRC5 to CCRT

The potential sensitization of chemo-radioresistant cells by nelfinavir was evaluated by exposing SiHa-P and SiHa-CRC5 cells to 20 μM nelfinavir for 24 h, followed by cisplatin or IR. Nelfinavir significantly reduced IC_{50}-value of cisplatin from 23.13 ± 1.78 μM to 10.42 ± 2.40 μM in SiHa-P cells, and from 35.31 ± 3.80 μM to 25.25 ± 1.82 μM in SiHa-CRC5 cells (Fig. 2A and B). Similarly, D0 dose was reduced from 2.28 to 1.50 Gy in SiHa-P cells, and from 3.12 to 0.89 Gy in SiHa-CRC5 cells (Fig. 2C and D). These results suggest that nelfinavir effectively enhances the sensitivity of both parental and resistant cells to chemo-radiotherapy.

3.5. Nelfinavir decreases HPV16 E6/E7 levels in chemo-radioresistant SiHa-CRC5 cells via downregulation of elevated USP15 and USP11

Elevated levels of HPV16 E6/E7 proteins observed in chemo-radioresistant sublines implied their role in resistance. Literature suggests that deubiquitinases (DUB's) USP15 and USP11 contribute to stabilization of E6 and E7 proteins, respectively [20,21]. Assessment of these deubiquitinases revealed significant upregulation of USP15 and USP11 in chemo-radioresistant sublines compared to parent cells (Fig. 2E and F). To investigate nelfinavir's effect on onco-proteins E6 and E7, SiHa-P and SiHa-CRC5 cells were treated with varying concentrations of nelfinavir. Both cell lines exhibited significant reductions in E6 and E7 protein levels (Fig. 2G–T). Importantly, nelfinavir treatment led to a significant reduction in USP15 and USP11 levels in both SiHa-P and SiHa-CRC5 cells (Fig. 2G–T), indicating that nelfinavir targets these deubiquitinases to reduce E6 and E7 expression.

3.6. Nelfinavir sensitizes chemo-radioresistant SiHa cells via inhibition of the AKT-USP15/USP11-HPV16 E6/E7 pathway

Nelfinavir, a known AKT inhibitor, was further evaluated for its effect on AKT signalling in both SiHa-P and SiHa-CRC5 cells. A significant

Table 1
IC_{50}-value and D0 dose for parent and chemo-radioresistant sublines.

Cell lines	Cisplatin IC_{50} (μM)	RI	p-value (IC_{50})	Cisplatin + IR IC_{50} (μM)	RI	p-value (IC_{50})	D0 dose (Gy)	RI	p-value (D0)
SiHa-P	23.13 ± 1.78	–		22.31 ± 1.87	–		2.28		
SiHa-CRC1	13.92 ± 1.85	0.59	<0.05	11.09 ± 1.32	0.49	<0.0005	1.81	0.79	<0.05
SiHa-CRC3	24.16 ± 0.92	1.04	ns	20.89 ± 0.95	0.93	ns	3.45	1.51	<0.0001
SiHa-CRC5	35.31 ± 3.80	1.52	<0.01	29.59 ± 3.12	1.32	<0.01	3.12	1.36	<0.0001

IC_{50}: Inhibitory concentration, RI: Resistance Index.

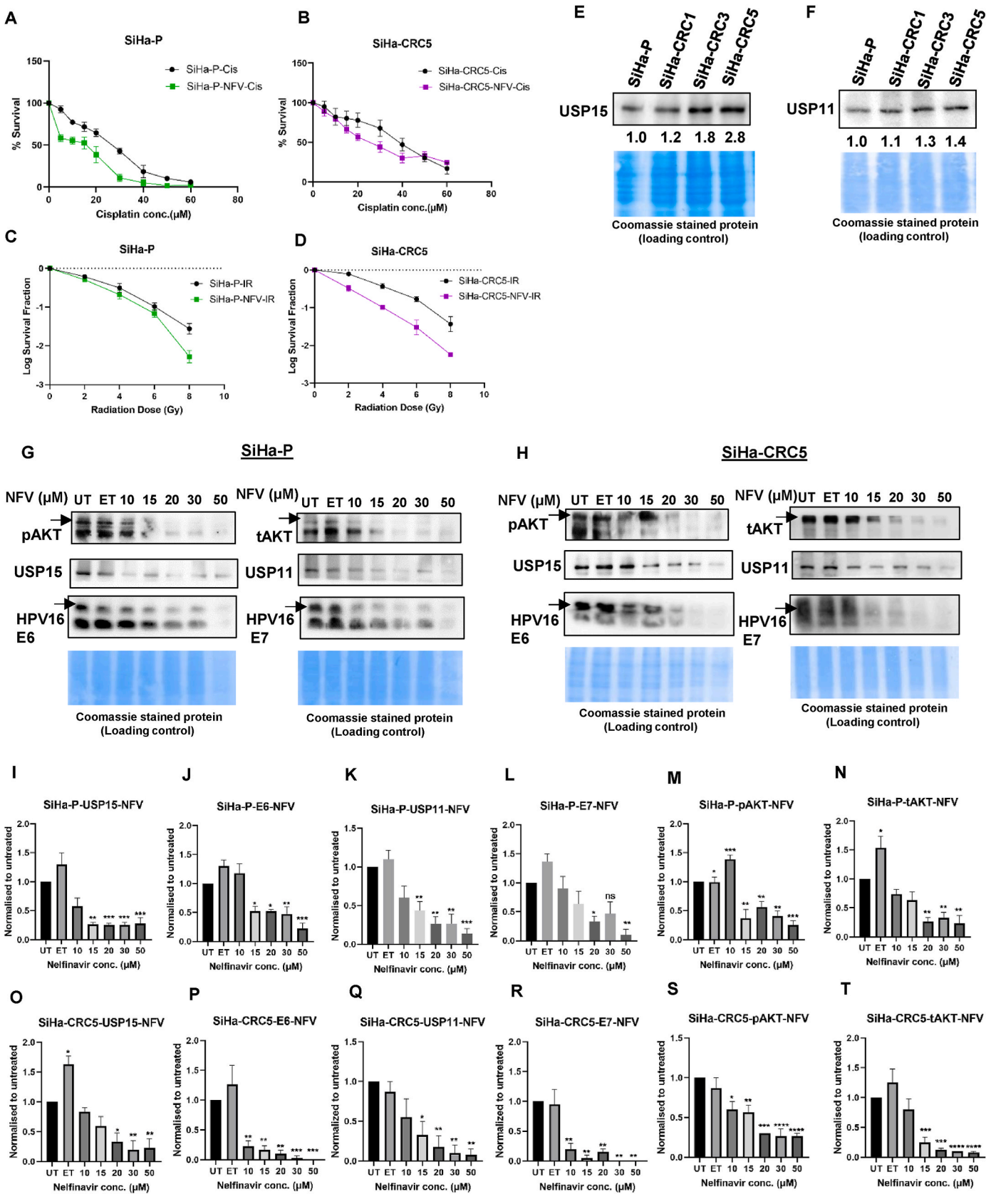

Fig. 2. Evaluate nelfinavir's effect on chemo-radioresistance: **(A&B)** Cisplatin IC_{50} values were determined by measuring percent cell survival, and **(C&D)** log survival fractions were plotted for SiHa-P and SiHa-CRC5 cells post nelfinavir treatment (20μM/24h), followed by cisplatin or IR using MTT and clonogenic assays. **(E&F)** Assessment of USP15 and USP11 expression between parental and chemo-radioresistant sublines. **(G–T)** Expression of HPV16 E6/E7, USP15/USP11, and AKT in SiHa-P and SiHa-CRC5 cells after treatment with gradient concentrations of nelfinavir (24 h). Densitometric analysis shows normalized protein expression as mean ± SEM.

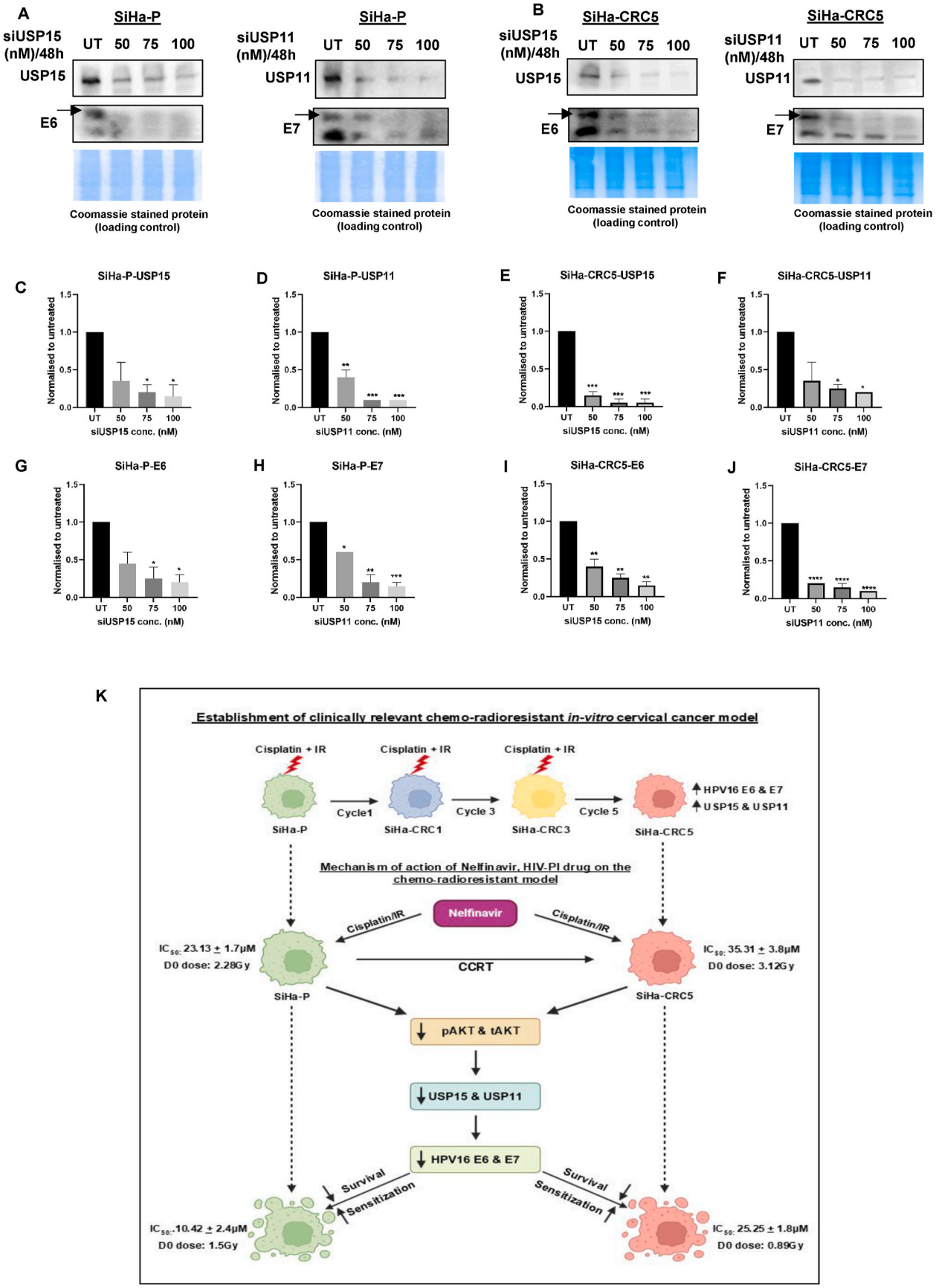

Fig. 3. Understanding nelfinavir's mechanism of action: **(A–J)** Expression of HPV16 E6 and E7 after siRNA-mediated knockdown of USP15 and USP11 in SiHa-P and SiHa-CRC5 cells. Densitometric analysis shows normalized protein expression as mean ± SEM. **(K)** Graphical illustration of nelfinavir's mechanism overcoming chemo-radioresistance in established model.

reduction in AKT protein and phosphorylated AKT (Ser-473) was observed following nelfinavir treatment (Fig. 2G–T). Like nelfinavir, treatment with AKT inhibitor IV also led to significant reductions in AKT, USP15, USP11, and HPV16 E6/E7 protein levels (Figs. S1A–N), further validating nelfinavir's mechanism of action. Furthermore, to decipher whether nelfinavir targets these deubiquitinases to reduce E6 and E7 expression, siRNA-mediated knockdown of USP15 and USP11 was performed in SiHa-CRC5 and SiHa-P cells which resulted in a significant reduction of E6 and E7 proteins respectively (Fig. 3A–J). Overall, these findings suggest that nelfinavir overcomes chemo-radioresistance by inhibiting the AKT-USP15/USP11-HPV16 E6/E7 signalling pathway (Fig. 3K).

4. Discussion

Concurrent chemoradiotherapy remains the cornerstone for treating locally advanced cervical cancer. While many patients initially respond to CCRT, disease recurrence, particularly at distant sites, is common [5,22]. The lack of effective salvage therapies after recurrence poses a significant clinical challenge in improving the clinical outcomes. Furthermore, devising novel therapeutic options necessitates the development of a robust, clinically relevant platform that can be used to improve therapeutic outcomes in this challenging cancer.

To model this clinical scenario, we developed CCRT-treated HPV16-positive cervical cancer SiHa sublines (CRC1, CRC3, CRC5), mirroring patient treatment regimens. Our data showed that the IC_{50}-values for these sublines reflect the clinical trend which is initial sensitivity to CCRT, followed by the development of resistance over successive treatment cycles. The progressive increase in resistance index from SiHa-CRC1 to SiHa-CRC5, indicates fundamental differences among these sublines and confirms prior studies indicating that sequential therapies induce resistance in cancer cells [23]. The significant survival advantage of SiHa-CRC5, even at higher cisplatin concentrations and post-irradiation, emphasizes the robustness of this model for studying therapy resistance. Although the RI of SiHa-CRC5 was not exceptionally high, it aligned with previously reported RI ranges of 1.0–16.1-fold, where models developed highly resistant cells through prolonged drug or radiation exposure but lacked clinical relevance [24–27]. Collectively, our data confirmed the successful development of a clinically relevant, chemo-radioresistant HPV16-positive cervical cancer model, which was not previously available.

Interestingly, previous studies have highlighted several genetic and epigenetic changes in resistance development such as elevated expression of multidrug resistance associated protein MRP2, overexpression of excision repair cross-complementing 1 (ERCC1) endonuclease, hypermethylation of DNA mismatch repair gene mutL homolog 1 (MLH1) and insulin-like growth factor binding protein-3 (IGFBP3) promoter [28]. Intriguingly our study demonstrated significant upregulation of HPV16 E6/E7 mRNA and protein implying the role of HPV-16 E6 and E7 oncoproteins in the resistance mechanism. Additionally, the observed unaltered HPV16 DNA copy numbers suggested that resistance in this model is not mediated by alterations in HPV viral load. Our observations align with earlier reports which imply that E6 and E7 promote resistance by enhancing DNA repair mechanisms and modulating apoptosis pathways [29].Specifically, E6-mediated degradation of p53 likely compromises the cellular response to DNA damage induced by chemoradiotherapy, thereby facilitating survival and therefore resistance. Similarly, E7's interaction with Rb and its effect on cell cycle dysregulation could contribute to an enhanced proliferative capacity despite therapy [29]. Additionally, Hampson et al. have reported high expression of HPV16 E6 oncoprotein in tumors generated from transplanted human cervical cancer cells C33A, to be associated with aggressive and radioresistant phenotype. This occurred through upregulation of ERCC1, a DNA repair enzyme [30]. Furthermore, Ma et al. demonstrated that E6/E7 induced 5-Florouracil resistance, through upregulated AKT-glycolytic pathway using *in-vitro* and *in-vivo* cervical cancer xenograft models [31]. Additionally, Jung et al. demonstrated *in-vitro* and *in-vivo* radiosensitization of cervical cancer cells on siRNA mediated silencing of E6/E7 [32]. However, there is limited information regarding changes in E6/E7 expression during sequential CCRT cycles and its impact on treatment response. Our study demonstrated significant upregulation of HPV16 E6/E7 oncoproteins post 1st, 3rd, and 5th CCRT cycles. As different E6 and E7 isoforms (11-21kDA) have been reported, the commonly observed E6/E7 protein band at ~16 kDa was considered for analysis [33,34]. Recently, Cosper et al. demonstrated that reduced immune response and persistent HPV E6/E7 gene expression post 3rd CCRT cycle contributed to resistance and mortality in cervical cancer [35], thus confirm our findings.

Nelfinavir, initially developed as an antiretroviral agent has demonstrated significant promise as an anticancer agent, primarily due to its ability to inhibit the AKT pathway [36]. This protease inhibitor has been reported to exhibit cytotoxic effects against multiple cancer, including non-small cell lung cancer, breast cancer (trastuzumab/tamoxifen-sensitive and -resistant) and cervix cancer (cisplatin-resistant and sensitive) through induction of endoplasmic reticulum (ER) stress, autophagy, and apoptosis in both *in-vitro* and *in-vivo* studies. [36,37]. While in our study the reduction in cisplatin's IC_{50}-value and D0 dose of irradiation levels suggests nelfinavir's potential for minimizing treatment-associated toxicities. This is particularly significant in the context of cervical cancer, where therapy-related side effects substantially impact quality of life [38].

Furthermore, Gupta et al. demonstrated that nelfinavir inhibited the growth of radiation-resistant human laryngeal squamous carcinoma cells (SQ20B) by reducing phosphorylated AKT levels. This effect was mediated via the induction of GADD34, a stress-inducible protein that promotes protein dephosphorylation, and the subsequent activation of protein phosphatase 1 (PP1), which dephosphorylates AKT and other substrates [39]. In our study, we observed that nelfinavir reduced both total AKT and phosphorylated AKT levels in chemo-radioresistant cervical cancer cells. These findings are consistent with those of Soprano et al., who reported that nelfinavir disrupted the AKT/HSP90 complex in breast cancer cells, leading to the downregulation of total and phosphorylated AKT [40].

Additionally, studies have highlighted the role of AKT in regulating deubiquitinases and vice versa. For instance, Liu et al. demonstrated that TGF-β activates the PI3K/AKT pathway, enhancing USP15 translation through a non-Smad-dependent mechanism [41]. Conversely, Yu et al. showed that USP15 negatively regulates AKT in degenerative nucleus pulposus tissue [42], a finding corroborated by Ren et al., who reported similar negative regulation of AKT by USP15 in hepatocellular carcinoma [43]. Similarly, Chen et al. reported that USP11 stabilizes eEF1A1, enhancing SP1-mediated activation of the HGF promoter and driving the PI3K/AKT pathway in hepatocellular carcinoma [44]. Interestingly, Park et al. demonstrated USP11's role in regulating PTEN polyubiquitination and stability. For instance, activated PTEN downregulates the PI3K/AKT pathway through PIP3 dephosphorylation in prostate cancer and non-cancerous systems [45]. Conversely in a total brain injury *in-vivo* model, Fang et al. demonstrated that USP11 negatively regulates the PI3K/AKT pathway by elevating Pyruvate Kinase M2 (PKM2) levels, causing neurological impairment and neuronal apoptosis [46]. However, our study revealed that nelfinavir induced positive regulation of both USP15 and USP11 via the PI3K/AKT pathway in parent SiHa-P and chemo-radioresistant SiHa CRC5 cervical cancer cells, which was not reported earlier. This observation underscores the complex interplay between nelfinavir treatment, DUB regulation, and the PI3K/AKT signalling pathway.

Furthermore, the ubiquitin-proteasome system has been shown to play a crucial role in regulating HPV oncoproteins [47]. USP15 and USP11 have been reported to directly interact with and deubiquitinate E6 and E7 respectively, stabilizing these proteins [20,21]. Previous studies have shown that nelfinavir can downregulate HPV E6/E7 post-translationally in CaSki cervical cancer cells [48]. Unlike previous

studies that linked nelfinavir primarily to AKT inhibition, we observed a reduction in phosphorylated AKT levels following nelfinavir treatment, with concomitant decrease in USP15 and USP11 levels, a previously unreported mechanism. This resulted in marked reduction in HPV E6/E7 oncoprotein levels. Thus, suggesting a direct link between AKT signalling and the stabilization of E6/E7 proteins. Moreover, siRNA-mediated knockdown of USP15 and USP11 independently reduced E6 and E7 protein levels, respectively, underscoring the therapeutic potential of targeting both viral and host-driven mechanisms. These findings align with studies indicating that targeting deubiquitinases can disrupt oncogenic pathways and sensitize tumors to therapy [49]. Mechanistically, our findings demonstrate that nelfinavir sensitizes chemo-radioresistant cells by targeting the AKT-USP15/USP11-HPV16 E6/E7 axis.

While the study provides compelling evidence for the role of HPV16 E6/E7 in the development of resistance to both the modalities (radiation and chemotherapy with cisplatin) and efficacy of nelfinavir in resistant cervical cancers, certain limitations warrant consideration. First, the *in-vitro* nature of the model may not fully capture the complexity of the tumor microenvironment. Validation in animal models and clinical samples is essential to corroborate these findings. Second, while the study highlights the role of USP15 and USP11, additional deubiquitinases or co-factors may contribute to E6/E7 stability. Comprehensive proteomic analyses could provide a broader understanding of the regulatory network involved. Finally, the potential off-target effects of nelfinavir and its long-term impact on normal tissues require further investigation. Combining nelfinavir with other targeted therapies or immunotherapeutic agents could enhance its specificity and efficacy. Future studies should focus on translating these insights into preclinical and clinical evaluations, advancing nelfinavir as a targeted therapy for HPV-associated cancers. Ongoing clinical trials are evaluating its efficacy in cancers treated with chemoradiotherapy, including glioblastomas, rectal & lung cancers. Notably, two registered trials (NCT03256916 and NCT01485731/NCT02363829) have investigated nelfinavir in cervical cancer, revealing promising outcomes [50]. Currently, a Phase III clinical trial is investigating nelfinavir's effects on AKT levels and disease-free survival in LACC patients undergoing CCRT and brachytherapy [51].

In conclusion, this study has established a robust *in-vitro* model for studying chemo-radioresistance in HPV16-positive cervical cancer and identified the AKT-USP15/USP11-HPV16 E6/E7 pathway as a critical driver of resistance. The findings highlight the potential of nelfinavir as a sensitizing agent, paving the way for its integration into therapeutic regimens. Further research is needed to translate these insights into clinical applications, to improve outcomes for patients with resistant cervical cancer.

CRediT authorship contribution statement

Reshma Reddy: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vagmi Gaiwak:** Validation, Investigation, Formal analysis. **Jayant Sastri Goda:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Tanuja Teni:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Informed Consent

Not applicable.

Ethics approval

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Data availability

Data will be made available on request.

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