

HIV-1 Suppresses Notch-1 Expression in HPV-16+ CaSki Cells for Cancer Progression

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Abstract

Background

High Risk Human Papilloma Viruses (HR-HPV) recurrently infect women having Human Immunodeficiency Virus – 1 (HIV-1)infection. Transforming HPV E6 and E7 genes promote invasive cancers and interact with Notch-1receptor. Concomitantly, HIV-1 Tat binds to EGF motifs within the Notch-1extracellular domain. HR-HPV infection activates Notch-1 signalling. Permissive HIV-1entry into the cervix is allowed. Notch-1 inhibitors may offer solace to the aggressive cancer phenotype in HIV-1 positive women. Still, the molecular cross talk between different oncogenes within the Notch-1pathway during HIV-1/HPV-16⁺ co-infections has not been elucidated.

Methods

Adherent cervical tumor derived cell lines-CaSki cell line (with inherent HPV16⁺ sequence and endogenous Notch-1 activity) and C33A cell line (cervical cancer phenotype, HPV -ve for HPV DNA and RNA) were used. Plasmids (pLEGFPN1 encoding HIV-1 Tat and pNL 4 – 3 encoding HIV-1(full HIV-1 genome), were transfected at 600 ng/mL into the above mentioned cell lines, in order to examine independent effects of HIV-1 Tat and HIV-1 transfection in HPV-16⁺ cervical cancer cells. We performed western blotting, cell cycle analysis and RT-qPCR post transfection. Three sets of independent experiments were analyzed by Graph Pad Prism 5. Statistical significance was calculated using Student t -test. Data expressed as mean \pm standard deviation (SD). p values \leq 0.05* were considered statistically significant.

Results

HIV-1 Tat and HIV-1 inhibited Notch-1expression, with differential effects on EGFR when comparing C33A and CaSki cell lines. CDK2 induction in Tat transfected CaSki cells, showed concomitant G₀/G₁ phase accumulation favouring cancer progression. Notch-1 inhibition shut off significant Cyclin D expression with a significant p21 induction and increased G₂-M cell population in CaSki cells. On the contrary, HIV-1 infection utilized Hes-1-EGFR-CyclinD-p21axis, G₂-M arrest, DDR response and cancer progression.

Conclusion

Our study highlights for the first time that HIV-1 Tat and/or HIV-1 driven cancers in HPV-16⁺ CaSki cells, show Notch-1 suppression and CDK2 dependent activity. HIV-1 Tat activates Hes-1 amplifying the EGFR gene which improves the aggressive state possibly through irreversible oxidative-stress induced senescence. HIV-1, favours cancer progression through its CXCR4 receptor, responsible for unbridled mitosis, G₂-M arrest and damaged DNA response (DDR). Treatment of CaSki cells with a Notch-1 inhibitor, DAPT showed marginal recovery in p21expression with G₀/G₁ and S phase recovery.

1. Background

There is an increased risk of co-infections with human papilloma virus (HPV) with persistence of many carcinogenic subtypes in Human Immunodeficiency Virus (HIV-1)-positive women [1]. They are more prone to invasive cervical cancers too [2, 3]. Epidemiological studies from a cohort of HIV-1 positive women established an incidence of 35.3 % carcinogenic human papillomavirus (HPV) genotypes. Oncogenic HPV-16 also escapes immune surveillance in HIV-1 positive women during combined antiretroviral therapy (cART) [4]. HPV vaccines have not yet been examined for the recurrence of cervical intraepithelial lesions in HIV-1 positive women [5].

HPV lifecycle orchestrates growth and differentiation events through HPV transforming genes-E6/E7 facilitating tumorigenesis [6]. During HIV-1 infection, the accessory protein *Tat* and HPV E6/E7 proteins exploit Notch signaling to exacerbate HPV-associated disease pathogenesis[7, 8]. Transmembrane Notch-1 protein is essential for proliferation, differentiation and apoptosis at all stages of life. Notch-1 activation, through ligand binding from neighboring cell encounter, induces proteolysis and release of Notch-1 intracellular domain (NICD) into the nucleus, the main gene processing centre [8]. Concomitantly, Numb, an evolutionary conserved protein enhances Notch-1 signaling by repressing NICD ubiquitination [9]. NICD also binds with DNA-binding transcription factor protein, CSL to initiate Notch-1 downstream targets, *Hes-1* and *Hey-1* gene transcription associated with carcinogenesis and progression of cervical carcinoma [10]. Alternative spliced Numb iso variants, short (Numb S) and long (Numb L) also influence *Hes-1* expression [10].

Notch-1, Numb and EGFR have similar roles in cell fate specifications including their involvement in cancers[11]. *In-vitro* studies in breast cancer cell lines demonstrated a positive link between Notch-1 and EGFR when transfected with HIV-1[12]. Notch-1 regulates growth and differentiation through cyclin D, CDK2 and p21 in cell lines like T-cell acute lymphoblastic leukemia [13]. Cervical cancer cells simultaneously utilize Notch-1[14] as well as hyper-express CXCR4, a co-receptor of HIV-1. The cross interference of intricate pathways converge together viz CXCR4/Notch-1, Wnt/Notch-1, Notch-1/Snail/Numb, Notch-1/EGFR etc are implicated in cancer progression, survival and chemotaxis via intracellular signaling /downstream mediators [15, 16].

Recurrent, invasive cervical carcinomas due to HIV-1 infection and interference with cell cycle marker expression, prompted us to examine the effects of HIV-1 *Tat* and full HIV-1 genome on HPV-ve C33A cells and HPV-16⁺ CaSki cells using transient transfection. We demonstrate here for the first time that both HIV-1 *Tat* and HIV-1 suppresses Notch-1 expression in CaSki (HPV-16⁺) cells through significant *Hes-1* induction and increased CDK2 expression for G₀-G₁ accumulation at the expense of S phase. HIV-1 *Tat* amplifies EGFR and p21 expression though Cyclin D is depleted, whereas HIV-1 transfection decreases EGFR and p21 expression concomitant with over active Cyclin D. Elevated Cyclin D preserves proliferation for unbridled mitosis, G₂-M arrest, genomic instability and cancer progression [17, 18]. Taken together, HIV-1 *Tat* shows a positive cross talk between Notch-1 and EGFR which favors an aggressive phenotype whilst HIV-1 has a reverse link between Notch-1 and EGFR. (Fig. 5)

2. Materials And Methods

2.1. Media and Experimental Reagents

The primary antibodies for Notch-1 (ab65297), Hes-1(ab49170), EGFR (ab2430), CDK2 (ab235941,) and GAPDH (sc32233) were purchased from Abcam, UK and SantaCruz, USA. The DNA transfection reagent, Jet PEI was procured from GeneX, India. The Notch-1 inhibitor-*N*[*N*-(3, 5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine t-butyl ester (DAPT) was obtained from Abcam, UK. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Antibiotic antimycotic solution (100×), Ethanol, 2-Mercaptoethanol and 2-Propanol (Isopropanol) were obtained from Sigma, USA. Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle Medium (DMEM) and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES-1M) were obtained from HiMedia Laboratories, India. The Fetal Bovine Serum (FBS) was obtained from Moregate, Australia. DNA was extracted using GF-1 Tissue DNA Extraction Kit from Vivantis Technologies, Malaysia. RNA isolation kit, Plasmid Miniprep Plus Purification Kit were procured from Gene Mark, USA. The Cycletest™ Plus DNA kit was procured from, BD Biosciences, USA. Power SYBR® Green RNA to CT™ 1-Step Kit was procured from Thermofisher, USA.

2.2 Cell lines

Adherent cervical tumor derived cell lines HPV-Negative C33A cells (HPV -ve for HPV DNA and RNA having cervical cancer phenotype)and HPV-16⁺ CaSki cells(with inherent HPV-16⁺ and HPV-18⁺ sequences and endogenous Notch-1), were used for transfection.

2.3. Cell culture

CaSki and C33A cell lines were procured from National Centre for Cell Sciences (NCCS), Pune. CaSki cell line was propagated in RPMI-1640 with sodium bicarbonate without L-Glutamine supplemented with 10 % fetal bovine serum and antibiotic, anti-mycotic solution. C33A cells were grown DMEM medium consisting of L-Glutamine, Sodium pyruvate, Glucose and Sodium bicarbonate.

2.4. Plasmids

The pLEGFPN1-(RV-Tat 86) plasmid was a gift from Dr. Francesca Peruzzi, Associate Professor, LSU Health Sciences, New Orleans LA. The pNL_{4,3}, a full length replication competent, infectious HIV-1 subtype B, was obtained through NIH AIDS Reagent Program.

2.4.1. Cell proliferation

Briefly, CaSki and C33A cells (10⁴/well) were seeded in 96micro well culture plates and maintained at 37°C in a CO₂ incubator. Cells were exposed to serial dilutions of plasmid DNA/DAPT and incubated overnight for 24 hrs. MTT solution (5 mg/mL) was added to the plate and then incubated for 4 hrs.

Supernatant was replaced with DMSO, and absorbance was measured at 550/630 nm. The viability was calculated in percentage with reference to control sets.

2.4.2. Recombinant cloning

Plasmids were transformed using DH-5 α through heat shock (42–45°C) and rapid chilling on ice. After heat shock treatment, the plasmid DH-5 α mixture was added to pre-warmed SOC or LB without antibiotics. LB agar plate containing ampicillin were used to grow the transformed bacteria, at 32°C. Colonies were further inoculated in 15 mL LB mixed with 65 % glycerol for stock preparation and plasmid amplification.

2.4.3 Plasmid Purification

Briefly, bacterial cells were pelleted, resuspended in 250 μ L of Solution I (as per the manufacturer's protocol-Gene Mark plasmid miniprep purification kit). With subsequent addition of 250 μ L Solution II, and following 4–5 times mixing, Solution III (250 μ L) was added. The spin columns containing lysate/s were centrifuged, rinsed and purged of purified DNA with preheated elution buffer. The DNA was quantified and stored at -20°C for transfection.

2.5. Transient Transfection

Transfections were carried according to the manufacturer's instructions (jetPEI® DNA transfection reagent, USA). Briefly, CaSki and C33A cells were seeded in T-25 flasks (1 X 10⁶) with 5 ml medium containing FCS and antibiotics. Quantified DNA-Six hundred ng/mL DNA was used for transfecting one million cells[19]. The media was replenished the next day and cell lines maintained overnight in a CO₂ incubator. Cells were detached for further analysis.

2.6. Cell cycle analysis

Transfected cells were scraped gently, washed twice in cold PBS and processed using BD Cycletest™ Plus DNA Kit as per the manufacturer's protocol. Briefly, cells were pelleted, mixed with Solution A (trypsin buffer) for 10 min followed by incubation with Solution B (trypsin inhibitor and RNase buffer) for 10 min. Finally the cells were stained with 200 μ L of cold Solution C (PI stain solution) kept for 10 min in the dark at 4 °C and analyzed for DNA content by BD FACSAria™ (BD, USA).

2.7. DNA isolation

DNA from transfected cells was isolated using the manufacturer's protocol (Vivantis Technologies, Malaysia). Briefly, the cell pellet (5 X 10⁶ cells) was resuspended in PBS, treated with Proteinase K and lysis enhancer in Tris buffer. After incubation at 65 °C for 10 min the cells were transferred to the column, followed by column washing with wash buffer. DNA was eluted with preheated elution buffer and quantified using spectrophotometer.

2.8. Polymerase Chain Reaction (PCR)

Notch-1 PCR was optimized with DNA isolated from human fetal buccal mucosal (FBM) cell line procured from ACTREC, Navi Mumbai, India. Twenty μL reaction mix (1.2 μL DNA template, primer mix and PCR dye master mix (5Xia)) was subjected to the following conditions ; 95 °C for 4 min, 45 cycles at 95 °C for 20 secs, 60 °C for 20 secs, two extension steps – 72 °C for 20 secs and 2 mins. Notch-1 PCR protocol was also used for CaSki cells.

Tat PCR was performed with DNA isolated from CaSki and C33A cells using the following conditions; 95 °C for 9 mins followed by 34 cycles at 95 °C for 1 min, 53 °C for 2 mins, two extension steps at 72 °C for 1 min and 7 mins respectively. PCR products were resolved on a 2.0 % agarose gel and visualized on a UV transilluminator after staining with ethidium bromide.

2.9. Western blot analysis

C33A and CaSki cells were harvested, washed, and lysed with RIPA buffer supplemented with phenylmethylsulfonyl fluoride (PMSF). Protein extracts (30 μg) per sample were subjected to 8 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, USA). Membranes were blocked with 5 % nonfat skim milk for 1 h at room temperature, probed with the appropriate primary antibodies (Notch-1, Hes-1, CDK2, EGFR and GAPDH a endogenous protein) at 1:1000 dilution, washed, and then incubated with the corresponding goat anti-rabbit and anti-mouse secondary antibody (1:5000 dilution). The chemiluminescent signal was detected using ECL system (Intron, Seongnam, Korea). Semi-quantitative analyses of the intensities of the protein bands were analyzed by using ImageJ software (NIH).

2.10. RNA Isolation

RNA was isolated from transfected cell lines as per the manufacturer's protocol (GeneMark Biolab, Thailand). Briefly, transfected culture cells were lysed with lysis methanol cocktail in 70 % ethanol. The lysate was loaded on the spin column and washed. After DNase treatment, the columns were washed again with wash buffer. Purified RNA (30–50 μL) was collected, quantified by a NanoDrop 2000c (Thermo Fisher Scientific, USA) and processed for qPCR.

2.11. Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was conducted using Applied Biosystems™ Power SYBR™ Green RNA-to-CT™ One-Step Kit and ABI 7500 FAST v2.3 (ThermoFisher, Singapore) as per the manufacturer's instructions. The qRT-PCR master mix (10 μL) consisted of a cocktail of 5 μL of 2X SYBR Green Real-time PCR Master, 2 μL primers - forward and reverse, 0.08 μL , RNA template (up to 100 ng), 2 μL , RNase free water and 0.92 μL , RT Enzyme (125 X). The thermal cycling conditions were 48 °C for 30 mins, followed by 1 cycle at 95 °C for 10 min, 45 cycles at 95°C for 15 sec, 60 °C for 10 secs, and 72 °C for 30 secs. Experiments were run in triplicates with β -actin as housekeeping gene. Relative concentrations were calculated using 7500 FAST software v2.3. Cycle threshold (C_T) values of transfected C33A cell line (an HPV⁻ cell line) served as the internal control for normalizing sample variations. The forward and reverse primers used for qRT-PCR

were human Notch-1, Hes-1, Hey-1, cyclin D, CDK2, p21, EGFR, and β -actin represented in supplementary Table 1.

2.12. Densitometry and statistical analysis

The results were analyzed by Graph Pad Prism 5. Student *t*-test was used to determine the statistical significance of the data expressed as mean \pm standard deviation (SD). *p* values $\leq 0.05^*$ were considered statistically significant.

3. Results

3.1. Effect of HIV-1 Tat transfection on CaSki cells

HIV-1 *Tat* maintained the viability of C33A and CaSki cells at 37 °C. HIV-1 *Tat* significantly inhibited Notch-1 activity as observed by the downstream transcriptional activator, Hes-1 hyperexpression in CaSki cells (Fig. 1B, * $p \leq 0.05$). For WB analysis, four proteins (Notch-1, Hes-1, EGFR and CDK2) were normalized with the GAPDH protein. Protein levels were plotted as the densitometric ratio of CaSki + HIV-1 *Tat* (600 ng/mL) versus CaSki only (Fig. 3A). Hey-1, (Fig. 1C), the downstream Notch-1 transcriptional repressor was significantly suppressed following transfection with *Tat* (C33A mock vs CaSki mock * $p \leq 0.05$; C33A 600 ng/mL vs CaSki 600 ng/mL * $p \leq 0.05$). Although HIV-1 *Tat* suppressed Notch-1 expression, EGFR was significantly upregulated when C33A and CaSki cell lines were compared (Fig. 1D, * $p \leq 0.05$). CDK2 induction (Fig. 1F, * $p \leq 0.05$) in *Tat* transfected CaSki cells, showed concomitant G₀/G₁ phase accumulation (60.8 %; 66.20 % ; supplementary Table 2) when compared to HPV-ve C33A cells (55.9 %: 81.55 %, * $p \leq 0.05$). Cyclin D expression was significantly shut down in CaSki (HPV-16⁺) cells (Fig. 1E) which coincided with a significant p21 induction (Fig. 1E & G) as well as increased cell numbers in CaSki G₂-M (12.3 % ; supplementary Table 2) phase cells.

3.2. Notch-1 activation explains aberrant mitosis in CaSki cells during HIV-1 transfection

HIV-1 maintained the viability in C33A and HPV-16⁺ CaSki cells at 37 °C. Figure 2A and 2B shows significant inhibition of Notch-1 expression, though its downstream target Hes-1 was overexpressed in HPV-16⁺ CaSki cells (* $p \leq 0.05$) indicative of Notch-1 signalling. Notch-1 and Hes-1 proteins decreased and matched with the qPCR profile (Fig. 3B). HIV-1 also significantly inhibited Notch-1 transcriptional repressor Hey-1 (Fig. 2C). Interestingly, HIV-1 induced reverse expressions of EGFR (Fig. 2D) and Cyclin D (Fig. 2E) as that of HIV-1 *Tat* (Fig. 1D & 1E). EGFR inhibition affects G₁ phase of the cell cycle, whereas overactive Cyclin D permits cellular differentiation and cancer progression. Additionally, overactive Cyclin D showed hyper CDK2 (Fig. 2F) expression corroborating increased G₀/G₁ cell accumulation. EGFR and CDK2 (Fig. 3B) protein levels overlap with the qPCR data. HIV-1 transfection of CaSki cells showed significant G₀/G₁ phase cell accumulation ($p \leq 0.05$; 78.35 %; supplementary Table 3) compared with

C33A cells (60.5 %). A significant decrease in the G₂-M cell population (4.65%; * p < 0.05; supplementary Table 3) was observed in CaSki cells as compared to HPV- C33A cells (* p ≤ 0.05; supplementary Table 3). Consequent to G₂-M arrest (supplementary Table 3) which collates with p21(* p ≤ 0.05; Fig. 2G) shut down, HIV-1 transfection confirmed transcriptional Hes-1 mediated ~ p21 repression in a bHLH domain dependent manner plausibly promoting Cyclin D induction. Excess Cyclin D forming complexes with CDK2, DNA Damaged response (DDR), genomic instability, subsequent DNA repair, unscheduled aberrant mitosis and cancer progression.

3.3. Incubation with Notch 1 inhibitor rescued p21 expression

CaSki cells were incubated with 62.5 mM/L DAPT to assess the effects of Notch-1 blockade on proliferation of HPV-16⁺ CaSki cells. MTT assay showed suppression of Notch-1 which affected the growth and proliferation of CaSki cells (supplementary data). qPCR data demonstrated a significant decreased expression for Notch-1, Hes-1, Hey-1 and EGFR gene 24 hours post incubation of DAPT with CaSki cells (* p ≤ 0.05; Fig. 4A) as compared with solvent control. Cell cycle analysis also showed G₀/G₁ arrest in DAPT -treated CaSki cells (supplementary Table 4). Consistent with G₀/G₁ arrest, cyclin D (* p ≤ 0.05 ; Fig. 4B) was maintained, with significant p21 restoration- (* p ≤ 0.05 Fig. 4B), and significant appreciable CDK2 expression. (* p ≤ 0.05; Fig. 4B).

4. Discussion

Notch family genes, crucial to normal embryogenesis are also implicated in cancers [20]. In HIV-1 /HPV co-infected women with cervical carcinoma, extracellular HIV-1 *Tat* clinically correlates with an aggressive carcinoma phenotype [21]. In HPV associated cancers, Notch-1 inhibition induces malignant transformation [6, 22]. In our study, we report for the first time that HIV-1 *Tat* significantly elicited Notch-1 inhibition in HPV-16⁺ CaSki cells through activation of Notch-1 signaling pathway, characterized by downstream target genes- Hes-1 transcriptional activator, and Hey-1, transcriptional repressor. Hey-1, appears to be depleted in CaSki cells a possible characteristic of transformed cell lines. Based on our data, we propose that HIV-1 *Tat* plausibly binds to EGF repeats on the extracellular Notch-1 domain [22], consequently suppressing Notch-1 expression. Hes-1 and Hey-1 genes are involved in adhesion, invasion, angiogenesis and proliferative events during carcinogenesis [23, 24]. In T-cell leukemia, Hes-1 binds to the nuclear Cyclin D promoter to elicit Notch-1 signaling. But, Hes-1 induction and Cyclin D repression are obligatory for sustenance and cancer progression in T-cell leukemia. Our observations in HIV-1 *Tat* transfected HPV-16⁺ CaSki cells, corroborate the above studies [23, 25]. HIV-1 *Tat* significantly induces Hes-1 expression for Cyclin D shut down (*p ≤ 0.05), with suppression of EGFR. Now, EGFR, the transcriptional target of Notch-1 [26], cooperates with downstream targets Cyclin D and CDK2 for growth, proliferation and cell cycle progression [27]. Additionally, Cyclin D, affiliated as downstream target of multiple oncogenic pathways, is also implicated in G₁ cell cycle progression. Cell cycle analysis demonstrate that HIV-1 *Tat* significantly activates EGFR with consequent significant G₀/G₁ phase cell

accumulation ($* p \leq 0.05$; supplementary Table 2) in HPV-16⁺ CaSki cells as compared with the HPV-C33A cells. Recent evidences highlight that Cyclin D depletion disturbs oxidative balance in cancer cells. Subsequently, cancer cells succumb to high lethal oxidative stress inducing irreversible senescence [27]. Hence, HIV-1 *Tat* adjusts the tumor microenvironment to maintain the proliferative state to evade irreversible senescence through Hes-1 activation, EGFR and p21 amplification driving cell migration and invasion for an aggressive less therapeutic phenotype in HPV-16⁺ CaSki cells [28, 29]. The above qRT-PCR data significantly collates with cell cycle G₂-M phase population of HPV-16⁺ CaSki cells with HPV-C33A cells ($* p \leq 0.05$).

HIV-1, significantly ($** p \leq 0.005$) inhibits Notch-1 and Hey-1 expression though its downstream transcriptional activator Hes-1 ($* p \leq 0.05$) was over expressed. Concomitantly, an oscillatory gene expression between Hes-1 and EGFR following HIV-1 infection indicates a plausible crosstalk between Notch-1 and EGFR pathways [12, 25]. As reported earlier, Hes-1 and Cyclin D gene expressions manipulate cancer maintenance and progression [24]. EGFR suppression and significant hyperactive CDK2 corroborate with cell cycle data showing significant G₀/G₁ phase cellular accumulation/s at the expense of S phase (supplementary Table 3; $* p \leq 0.05$) for cancer progression. Excess CDK2 forms nuclear complexes with Cyclin D, disturbing cell cycle progression [27]. Significant CDK2 hyper expression ($* p \leq 0.05$) and subsequent p21 shut down ($* p \leq 0.05$; Fig. 2F, 2G), decline in polyploid G₂-M phase HPV-16⁺ CaSki cells (4.65%; $* p \leq 0.05$; supplementary Table 3) was observed (data not shown) [27, 28, 29]. HIV-1 Vpr (Viral Protein R) transcription causes G₂-M of the cell cycle arrest inhibiting cell proliferation [30]. Additive effects of HIV-1 Vpr and Notch-1/ CXCR4 [16] partner together to inhibit p21 and juxtapose aberrant mitosis, G₂-M arrest for induction of replication restart [31] for metastasis and cancer progression.

Our results with DAPT, a drug induced Notch-1 inhibitor and γ -secretase blocker revealed significant recovery in the G₀/G₁ and S phase HPV-16⁺ CaSki cells (supplementary Table 4). Notch-1 inhibition was approximately 35% ($* p \leq 0.05$; Fig. 4A). Downstream targets Hes-1 & Hey-1 were significantly inhibited ($* p \leq 0.05$; Fig. 4A). There was a marginal recovery in p21 expression ($* p \leq 0.05$; Fig. 4B). DAPT rescues G₀/G₁ ($* p \leq 0.05$; supplementary Table 4) and S phase ($* p \leq 0.05$; supplementary Table 4) during HPV carcinogenesis.

5. Conclusion

Our results describe a negative link between Notch-1 and EGFR during HIV-1 induced cancer progression. Tailor-made drug paradigms for clinical interventions may possibly inhibit an aggressive phenotype during cancer progression.

6. List Of Abbreviations

1	HIV1	Human Immunodeficiency Virus subtype 1
2	HPV	Human Papilloma Virus
3	E6/E7	Viral oncogenes (Early) of human papilloma virus
4	HR-HPV	High Risk Human Papilloma Virus
5	Hes-1	Transcriptional repressor- downstream target of Notch1 Hairy enhancer of Split-1
6	Hey-1	Hairy/enhancer-of-split related with YRPW motif protein 1
7	EGFR	Epidermal Growth Factor Receptor
8	CDK2	Cyclin Dependent Kinase-2
9	Tat	Transactivator of Transcription
10	Vpr	Viral Protein R
11	CXCR4	G-protein coupled chemokine receptor used by HIV to infect CD4 ⁺ T cells
12	CaSki cell line	Adherent epithelial cell line having inherent 600 copies of HPV-16 ⁺ and HPV-18 ⁺ sequences and with Notch-1 activity
13	C33A cell line	Adherent epithelial cell line having cervical cancer phenotype but HPV ⁻
14	FBM	Foetal Buccal Mucosal Cells
15	FBS	Foetal Bovine Serum
16	DAPT	N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester {Notch1 inhibitor}
17	DDR	Damaged DNA Response
18	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
19	DMEM	Dulbecco's Modified Eagle Medium
20	RPMI-1640	Roswell Park Memorial Institute
21	DMSO	Di methyl sulphoxide
22	SOC	Microbial growth medium used for the transformation of competent cells (E. coli)
23	LB broth	Lysogeny broth / Luria-Bertani medium
24	PBS	Phosphate buffered saline
25	PMSF	Phenylmethylsulfonyl fluoride

1	HIV1	Human Immunodeficiency Virus subtype 1
26	RT-PCR or qPCR	Real Time or quantitative Polymerase Chain Reaction
27	p21	A potent cyclin-dependent kinase inhibitor (CKI) which binds and inhibits CDK2, CDK1 activity, during cell cycle progression from G ₁ to S phase.
28	WB	Western blotting

7. Declarations

Ethics approval

ICMR-NARI institutional review board approved this research study. Waiver of consent (vide NARI/EC Protocol No.: 2015-15) for C33A and CaSki cell lines was also granted.

Consent for publication

No writing assistance was provided.

Availability of the data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files. This information are available on request.

Competing interest

All authors have no conflicting interests to declare.

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Authors contribution

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II. Experiments performed:

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b. *Gel electrophoresis*: Mrs Linata Patil

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- e. *Transfection of cell lines: Dr Serena D'Souza,*
- f. *DNA isolation from mock and transfected cell lines: Mrs Linata Patil, Dr Serena D'Souza, Dr Aarti Mane*
- g. *RNA isolation, quantitation and gel electrophoresis for RNA integrity : Dr Serena D'Souza*
- h. *RT-PCR: Dr Serena D'Souza, Dr Madhuri Thakar*
- i. *Experiments with Notch-1 inhibitor DAPT: Ms Leila Fotooh Abadi, Dr Serena D'Souza, Dr Samiran Panda*
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9. Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

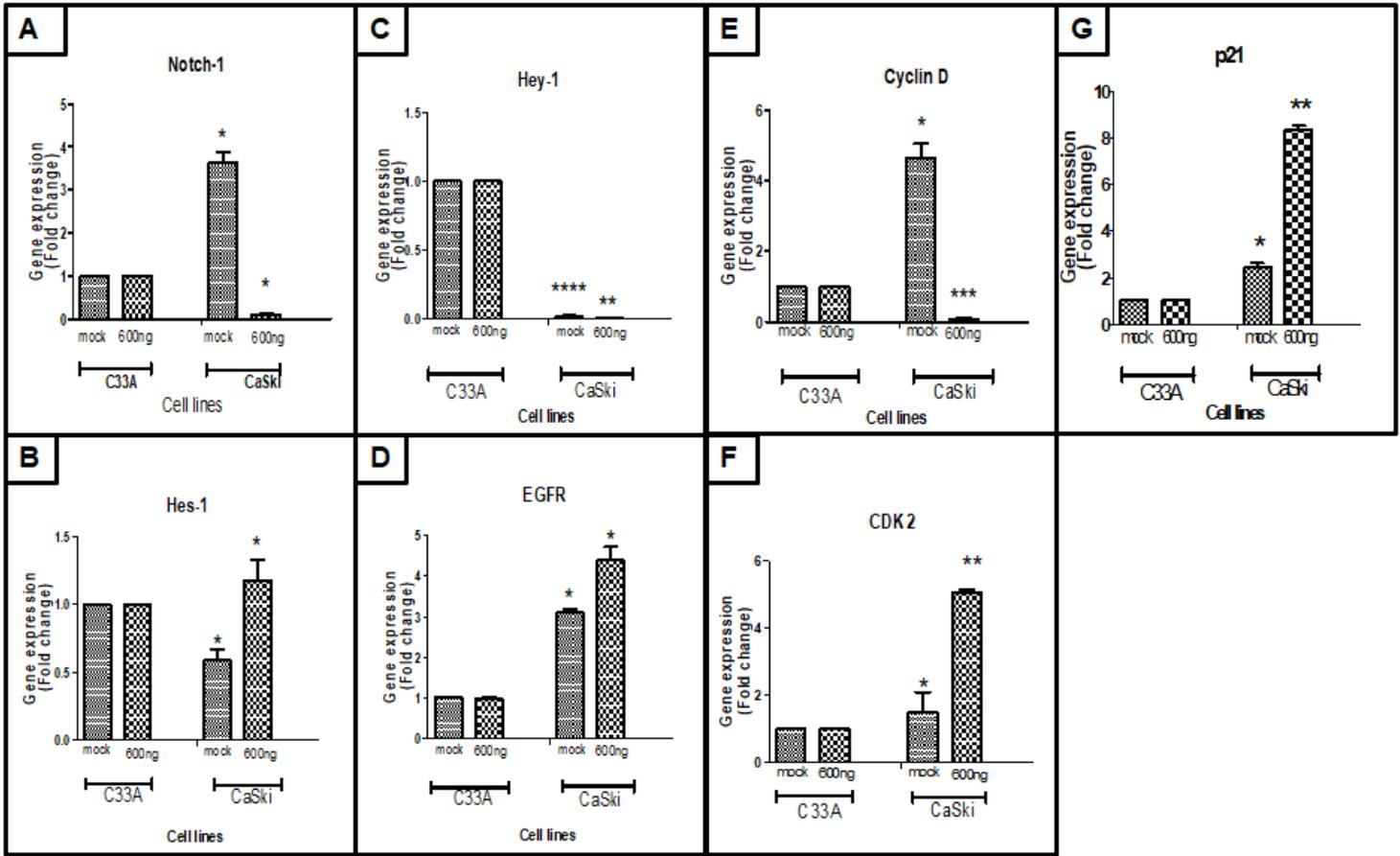


Figure 1

Relative gene expression of HPV-16+ CaSki Cells normalized with HPV negative C33A cells after transfection with Tat DNA. A-G : The x-axis shows transfected cell lines with Tat DNA. Mock represents solvent control. Fold change for the following genes was calculated using HPV-16 + CaSki cells (A)- Notch-1 and its transcription factors (B)-Hes-1 and (C)-Hey-1 (D)-EGFR (E) -Cyclin D (F)-CDK2 and (G)- p21. Level of significance *p<0.05 was calculated using Student's 't' test

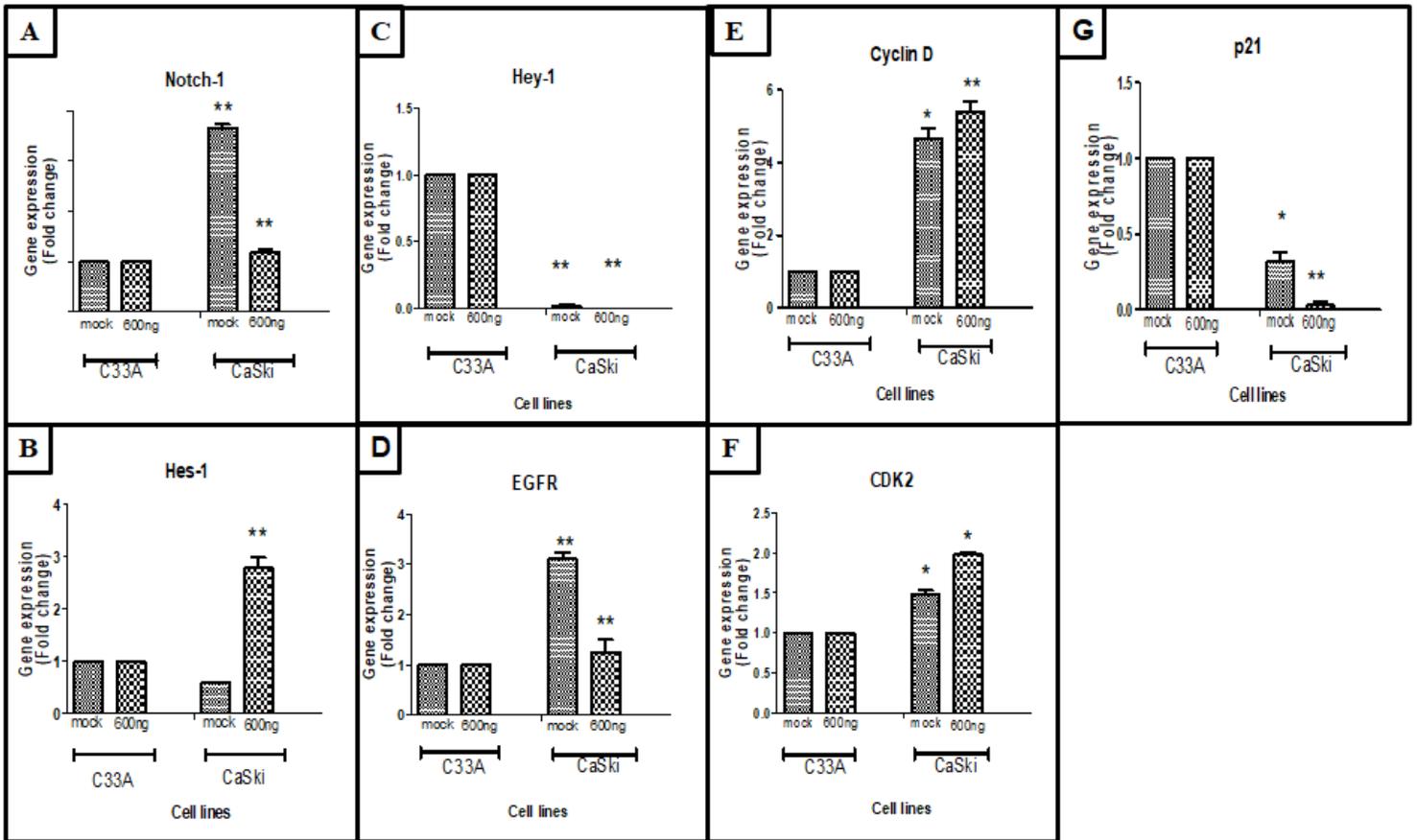


Figure 2

Relative gene expression of HPV-16+ CaSki Cells normalized with HPV negative C33A cells after transfection with HIV-1DNA. A-G : The x-axis shows transfected cell lines with HIV-1 DNA. Mock represents solvent control. Fold change for the following genes was calculated using HPV-16 + CaSki cells (A)-Notch-1 and its transcription factors (B)-Hes-1 and (C)-Hey-1 (D)-EGFR (E) -Cyclin D (F)-CDK2 and (G)-p21. Level of significance *p<0.05 was calculated using Student's 't' test

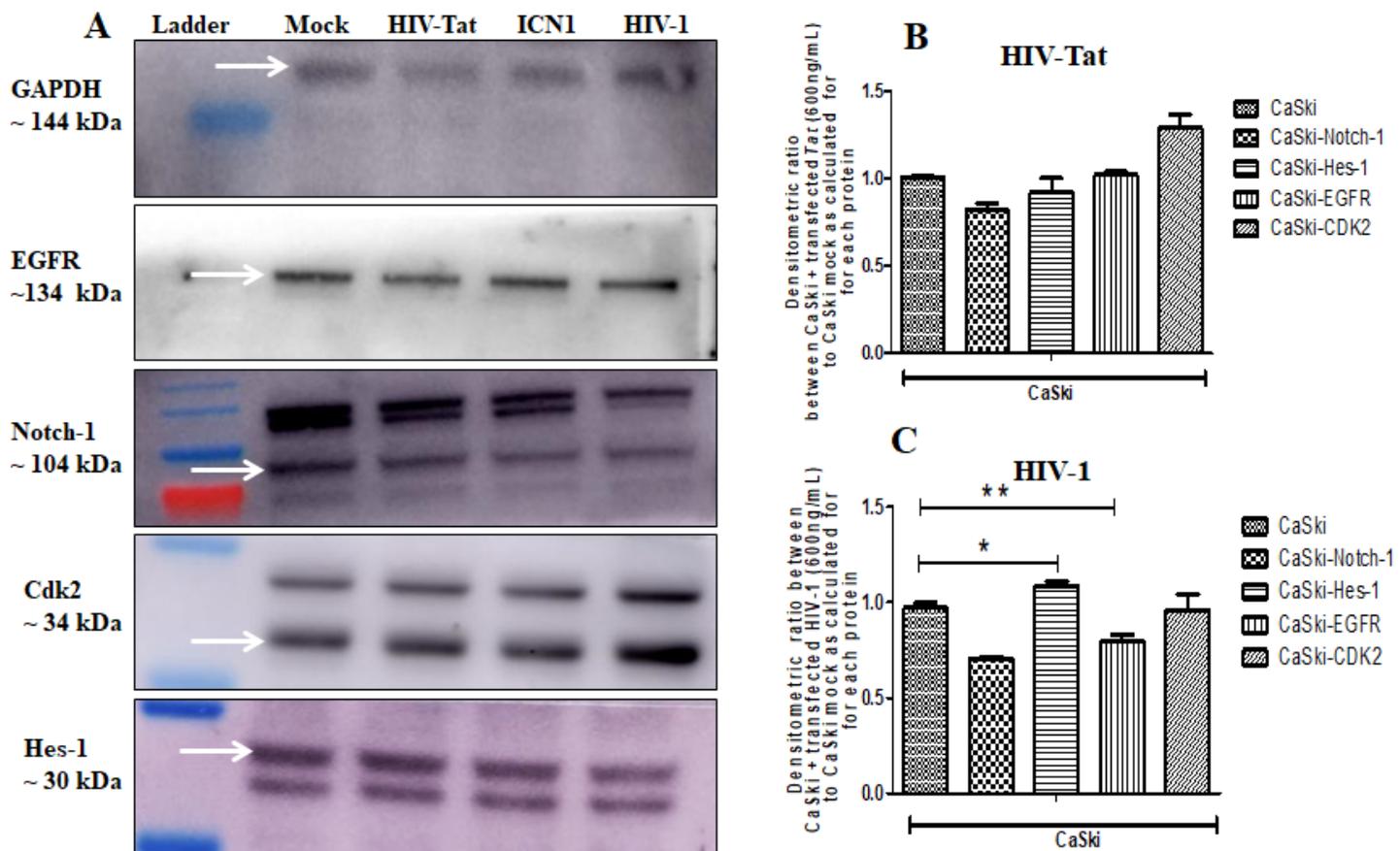


Figure 3

A: Western blotting analysis to assess band density using Image J software for four proteins. GAPDH served as the house keeping gene. Intracellular Notch-1 (ICN1) was taken as positive control. B: Densitometric ratio of HIV-1 Tat transfected CaSki cells (600ng/mL) compared with CaSki mock. Level of significance $*p < 0.05$ was calculated using Student's 't' test. C: Densitometric ratio of HIV-1 transfected CaSki cells (600ng/mL) compared with CaSki mock. Level of significance $*p < 0.05$ was calculated using Student's 't' test

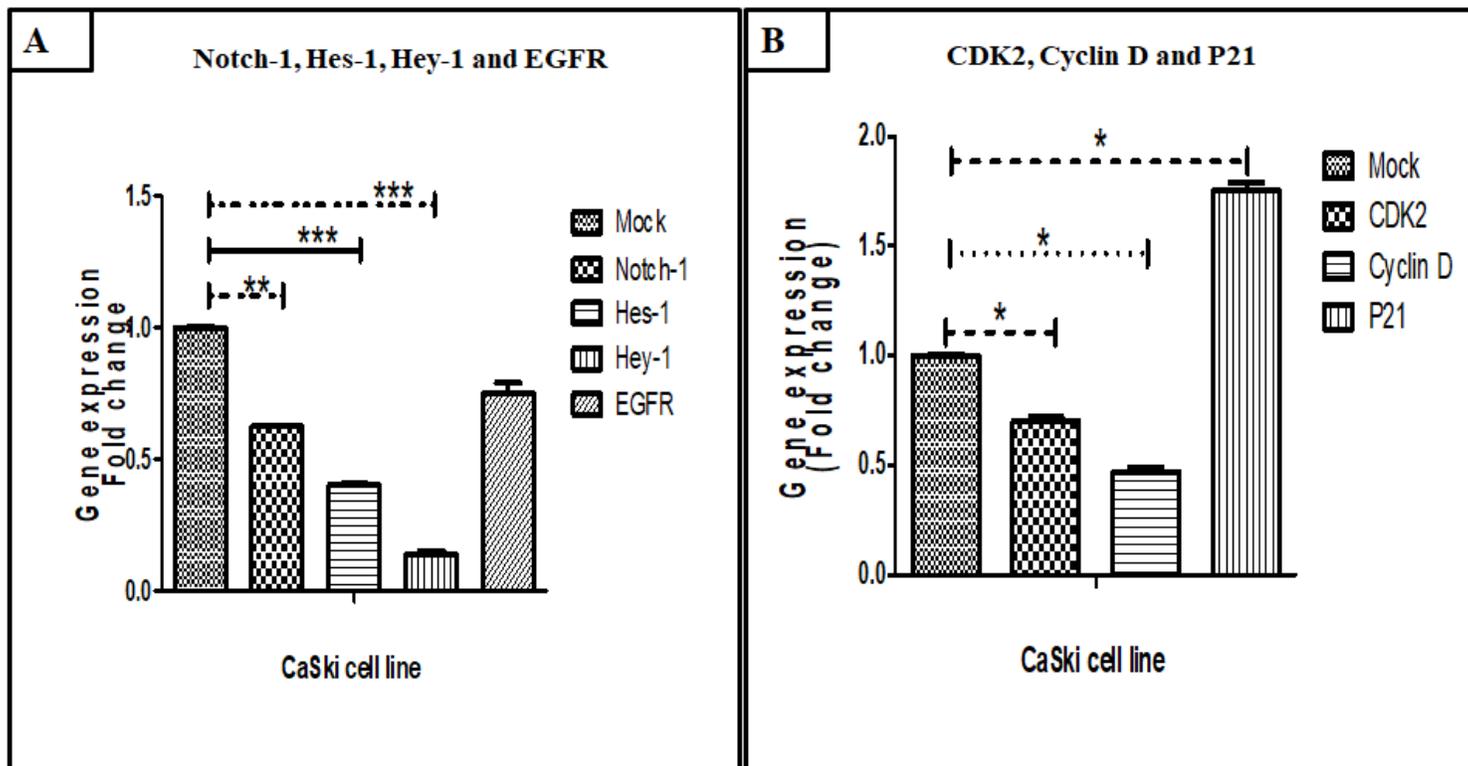


Figure 4

A : Mock represents solvent control used in preparation of 62.5 mM/LDAPT, a Notch-1 inhibitor. X-axis represents the relative gene expression (fold change) for mock, Notch-1, Hes-1, Hey-1 & EGFR. Level of significance $*p < 0.05$ was calculated using Student's 't' test. B : Mock represents solvent control used in preparation of 62.5 mM/LDAPT, a Notch-1 inhibitor. X-axis represents the relative gene expression (fold change) for mock, cyclin D, CDK2 & p21. Level of significance $*p < 0.05$ was calculated using Student's 't' test

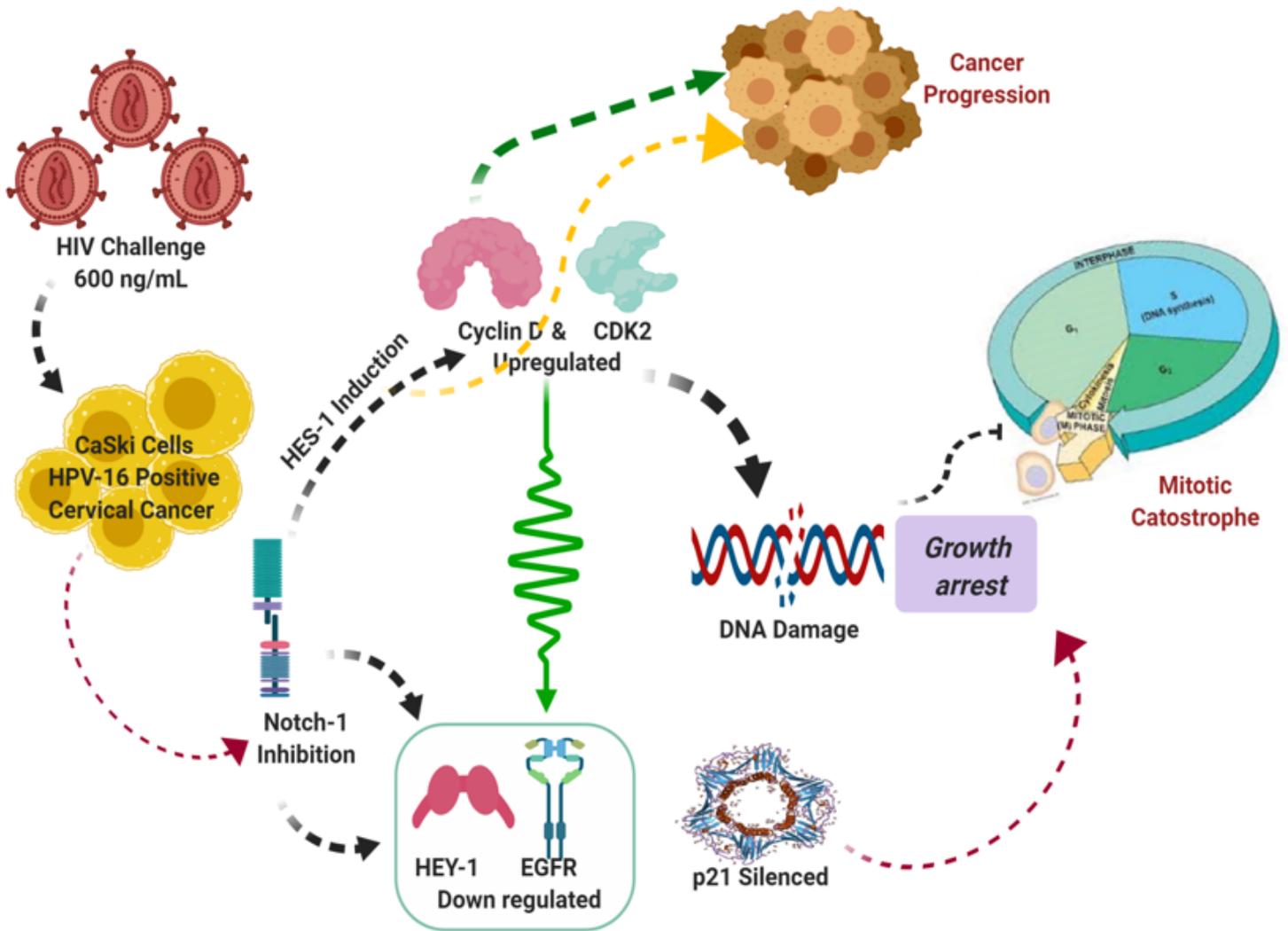


Figure 5

Graphical Abstract showing how HIV interacts with HPV16 to induce Notch-1 suppression for cancer progression. (Created with BioRender.com)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TABLES.pptx
- SUPPLEMENTARYDATA24032021.pptx