RESEARCH ARTICLE

Reversal of Resistance towards Cisplatin by Curcumin in Cervical Cancer Cells

Madhumita Roy[1]*, Sutapa Mukherjee[2]

Abstract

Epigenetic regulators like histone deacetylases (1 and 2), and viral onco-proteins (E6/E7) are known to be overexpressed in cervical cancer cells. The present study was designed to investigate the effect of curcumin on HDACs (1 and 2) and HPV E6/E7 in the cervical cancer cell line SiHa and a drug resistant clone SiHa^R (derived from SiHa). It was further intended to investigate whether curcumin could sensitize the cells towards cisplatin induced cell killing by modulation of multi drug resistant proteins like MRP1 and Pgp1. Curcumin inhibited HDACs, HPV expression and differentially increased acetylation and up-regulation of p53 in SiHa and SiHa^R, leading to cell cycle arrest at G1-S phase. Up-regulation of pRb, p21, p27 and corresponding inhibition of cyclin D1 and CDK4 were observed. Cisplatin resistance in SiHa^R due to over-expression of MRP1 and Pgp1 was overcome by curcumin. Curcumin also sensitized both the cervical cancer cells towards cisplatin induced cell killing. Inhibition of HDACs and HPVs led to cell cycle arrest at G1/S phase by alteration of cell cycle regulatory proteins. Suppression of MRP1 and Pgp1 by curcumin resulted in sensitization of cervical cancer cells, lowering the chemotherapeutic dose of the drug cisplatin.

Keywords: HDACs - E6/E7 - MDR - cisplatin - cervical cancer

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Introduction

Cervical cancer is the third most common cancer in women and almost all cervical cancers are caused by HPV (human papilloma virus), which is a common virus that is spread through sexual intercourse. Human papilloma virus (HPV) particularly HPV (16 and 18) are the primary causative agents of cervical cancer (Lin et al., 2009) as HPV-DNA was detected in majority of the cervical cancer cases (Raybould et al., 2011). Viral DNA integration into the host cell results in disruption of expression of viral gene; deregulation of E6 and E7 on the other hand may culminate into harmful effects (Cheah et al, 2012). E6 and E7 oncoproteins are involved in aberrant proliferation and increased expression of malignant phenotype in cervical epithelial cells (Mockel et al., 2011). HPV gets integrated into the host genome thereby deregulating host tumor suppressor proteins p53 and pRb via E6 and E7 proteins (Cheah et al, 2012). HPV-E6 protein forms a complex with E6-AP and subsequently interacts with p53, leading to ubiquitin dependent proteasomal degradation (Subramanian and Chinnappan, 2013).

Stabilization of p53 is being facilitated by acetylation at lysine residues by histone acetyl transferase (HAT), whereas de-acetylation by histone deacetylases (HDACs) leads to transcriptional repression and degradation of p53 (Lin et al., 2009). E7 oncoprotein binds with pRb (Zhang and Tang, 2012) and this retinoblastoma protein controls the G1-S transition in the cell cycle. Interaction of pRb and E7 disrupts and releases E2F (Zhang and Tang, 2012). pRb recruits HDAC and changes the chromatin structure at the E2F-responsive promoter, thus controls the transcriptional activity of E2F (Tanaki et al., 2004). Interaction of pRb with E2F is therefore crucial for repression of E2F-responsive proliferation associated genes, which gets altered in cervical cancer (Jayshree et al., 2009). E7 also binds with HDAC (1 and 2) through the CR3 zinc-finger domain, which is independent of pRb interaction (Brehm et al., 1999); this is important for immortalization and episomal maintenance of E7 protein (Longworth and Laimins, 2004). E7 furthermore forms complexes with cyclin dependent kinase inhibitors p21 and p27 and abrogates the function of these CDK inhibitors to override normal cell cycle checkpoints (Narisawa-Saito and Kiyono, 2007). HDAC (1 and 2) over-expression represses tumor suppressor genes (Takai et al., 2011) and their knock down can induce p21 expression; leading to induction of apoptosis in cervical cancer cells (Witt et al., 2009). Over-expression of HDAC2 has been observed in cervical cancer (Huang et al., 2005).

Therefore identification of compounds endowed with inhibition of HDACs and HPV-associated molecular events will be of enormous importance in cervical cancer control. Natural plant products elicit ideal cancer chemopreventive activities and therefore can be used for cancer control. Curcumin, a polyphenolic compound

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extracted from the rhizome part of the plant Curcuma longa is an Indian spice exhibiting chemopreventive properties (Biswas et al., 2010). Several preclinical and clinical studies indicated the anticancer potential of curcumin (Basu et al., 2013; Dai et al., 2013; Yin et al., 2013). Curcumin exhibited its antitumor action in HPV associated cervical cancer (Basu et al., 2013) by restoring p53 and pRb (Maher et al., 2011) and by reducing the level of cyclin D1 and activation of tumor suppressor proteins (Singh and Singh, 2011).

Drug resistance to chemotherapy is a major threat in cancer management. Multi drug resistance is considered to be a major cause of failure of conventional chemotherapy (Dong and Mumper, 2010). Some membrane proteins like P-glycoprotein 1 (Pgp1), multidrug resistance protein 1 (MRP1) belonging to ATP binding cassette (ABC) transporter family play important roles in the development of multidrug resistance (Kuo, 2009; Singh et al., 2012). Therefore overcoming drug resistance is a novel approach in cancer research. A drug resistant clone SiHa^R (resistant to cisplatin), derived from SiHa was used for this study. The study was conducted in SiHa and its drug resistant counterpart SiHa^R. Ect1/E6E7, a normal cervical epithelial cell line was used as a control. Present study was designed to examine the effect of curcumin on HDAC (1 and 2), E6/E7 viral oncoproteins, multidrug resistant proteins Pgp1 and MRP1 in these cell lines, with the aim to elucidate the mechanism involved there in. It was furthermore intended to investigate whether curcumin by virtue of modulation of these proteins could reverse platinum drug resistance and increase the sensitivity of cervical cancer cells to cisplatin.

Materials and Methods

Chemicals

Curcumin and Trichostatin A (TSA) was procured from Sigma Aldrich, USA. Nitrocellulose membrane from Hybond ECL, Amersham Biosciences, U.K and anti-HDAC1(05-614), anti-HDAC2(05-814), anti-acetyl-p53-Lys320(06-1283), anti-acetyl-p53-Lys373(04-1137), anti-acetyl-p53-Lys382(04-1146), anti-p53(05-224), HDAC assay kit(17-374) were obtained from Upstate, Millipore, Massachusetts, USA. Antibodies against HPV16-E6(sc-1584), HPV16-E7(sc-6981), from Santa Cruz Biotechnology, Inc, CA, USA and anti-p21(ab16767), anti-MRP1(ab3368), anti-Pgp1(ab103477), anti-p27KIP1(ab54563), anti-p73(5B1288) (ab22045), anti-Cyclin D1(ab10540), anti-CDK-4(ab7955), anti-β-actin(ab6276) and anti-histone H1(ab4269) were purchased from abcam, Cambridge, Massachusetts, USA. Kit for Glutathione estimation was obtained from Cayman (703002). Other reagents used were of analytical grade and procured locally. Chemotherapeutic drug cisplatin used in this study was purchased locally.

Isolation of cell line

A cell line was derived from SiHa by growing exponential cells in a stepwise increasing concentration of cisplatin (0.1 μg/ml-20 μg/ml). Cells were maintained in each concentration for 6 passages and finally a colony has been isolated at 20 μg/ml; this has been designated as SiHa^R.

Maintenance of Cell Lines

Human cervical cancer cell lines SiHa and SiHa^R were maintained in DMEM supplemented with 15% heat inactivated fetal bovine serum (FBS) and antibiotics (gentamycin 40 μg, penicillin 100 units, streptomycin 10 μg/ml). The normal cervical epithelial cell Ect1/E6E7 was maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% fetal bovine serum. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Characterization of SiHa^R

SiHa^R had been tested for the MRP1 and Pgp1 status and the corresponding GSH level. All these parameters had been checked in the parental SiHa to get a comparative estimate of drug resistance developed in SiHa^R.

Expression of HDAC (1 and 2) and HDAC activity assay

Expressions of HDAC (1 and 2) using specific antibodies were determined in curcumin treated cells by western blot analysis and HDAC activity was measured using assay kit (Roy et al., 2011). Briefly, treated cells were harvested, washed in wash buffer pH 7.5 (HEPES. KOH 10mM, $MgCl_2$ 1mM, KCl 10mM, DTT 1mM) and then lysed in lysis buffer (Tris.HCl 10μM pH 7.5, $MgCl_2$ 1mM, 0.065% β-mercaptoethanol, 0.5% CHAPS and 10% glycerol). The lysates were then centrifuged at 10,300 g for 20 min and protein concentration was quantified following Lowry's method. Protein was loaded in each well and then electrophoresed on 10% SDS-polyacrylamide gel using electrophoresis buffer (Tris 25 mM, glycine 192 mM, 10% SDS). Separated proteins were transferred to nitrocellulose membranes using transfer buffer (Tris 250 mM, glycine 192 mM, 10% methanol), followed by blocking in BSA. Membranes were washed with TBS solution pH 7.5 (Tris.HCl 25 mM and NaCl 150 mM). Immunodetection was done by incubating the membrane with primary antibodies overnight at 4°C with constant shaking followed by washing in TBST (TBS containing Tween-20) and then incubated with alkaline phosphatase conjugated anti-mouse IgG (1:1000 dilutions in TBS) at 4°C. Protein bands were visualized by treating the membrane with the substrate BCIP/NBT.

Expression of E6/E7 proteins, acetylated p53, p53, pRb, p21, p27, p73, Cyclin D1, CDK4, MRP1 and Pgp1

Cells cultured in presence and absence of curcumin for 24h were fractionated into nuclear and cytosolic fraction or total cell lysates were prepared following the method of Roy et al., 2011. Total cell lysates or the particulate fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes following the method as described for HDACs. Membranes were then probed with antibodies against HPV16 E6, HPV16 E7, acetylated p53, p53, pRb, p21, p27, p73, Cyclin D1, CDK4, MRP1 and Pgp1. Membrane was washed and incubated with alkaline phosphatase conjugated anti-mouse IgG/alkaline phosphatase conjugated anti-rabbit IgG (Genei, India) and

proteins were visualized by addition of substrate BCIP-NBT (Genei, India).

Cell Cycle analysis by flow cytometry

Treated cells were suspended in cold PBS and centrifuged at 100 g for 8 min. Cells ($2x10^6$) were fixed in 70% cold absolute ethanol incubated in ice (30 min), and then centrifuged to remove ethanol. Cell pellets were suspended in 1ml DNA binding solution (200 μg/ml RNase+50 μg/ml PI) and kept in dark (30 min) before analysis in Becton Dickinson Flow Cytometer, using a nitrogen argon laser. Fluorescence was captured on FL2H channel with logarithmic amplification. 10,000 cells were counted for each determination.

Estimation of glutathione

Glutathione assay was done following the protocol mentioned in the kit. Cell lysates were de-proteinated by adding an equal volume of metaphosphoric acid and mixed on a vortex. They were then kept at room temperature for 5 min, followed by centrifugation at >200 g for 2 min. The supernatant was carefully aspirated and stored at -20°C. During assay, samples were treated with 50μl of 4M triethanolamine solution. Standards of GSSG (25 μM) and MES (0.4M 2-(N-morpholino)ethane sulphonic acid, 0.1 M phosphate and 2mM EDTA, pH 6.0) buffer were prepared and this corresponded to GSH concentration between 0 and 16 μM, to get the standard curve. Standard and samples, in equal proportion were taken in triplicates in a 96 well plate; 150 μl of assay cocktail (freshly prepared) was added. The plate was kept in dark on an orbital shaker and finally absorbance was noted at 414 nm at 5 min interval for 30 min, using a TECAN Infinite M200 plate reader.

Assessment of cytotoxicity

Cytotoxic efficacy of cisplatin was determined by the MTT assay (Roy et al., 2011). Cells at a density of 104 were seeded in each of the 96 well plates and exponentially growing cells were treated with curcumin along with cisplatin of different concentrations (indicated in the Figure Legends) for 24 h. Cells treated with TSA (1 μM), a HDAC inhibitor along with cisplatin was considered as positive controls. MTT solution 50 μl (1.2 mg/ml in water) was added to each of the 96 wells and incubated for 4 h. Plate was centrifuged for 10 min at 140 g at 4°C. Supernatant was discarded and DMSO was added to dissolve MTT-formazan product (purple colored), which was estimated by measuring absorbance at 570 nm in an ELISA plate reader (Model: TECAN-Infinite M200).

Clonogenic survival assay

$2x10^6$ cells were seeded and exponentially growing cells were treated with 50 μM of curcumin along with different concentrations of cisplatin (0.1, 1.0, 10, 100 μM) for 24 h. Treated cells were trypsinized, harvested and centrifuged at 160 g for 6 min, followed by washing in PBS twice. Cells were counted and seeded and incubated till colony formation. Plates were stained with methylene blue and colonies of 50 cells or more were counted. Surviving fraction (SF) was determined using the formula: SF=ratio of colony number in treated cells to those of untreated cells.

Statistical calculation

Statistical calculations were carried out by paired sample t test.

Results

Constitutive expression of MRP1, Pgp1, HDAC (1 and 2) and E6 and E7 in SiHa, SiHa^R and normal Ect1/E6E7 cells

Expressions of MRP1 and Pgp1 were examined in Ect1/E6E7, SiHa and SiHa^R (in all the clones during development of drug resistance) cells. Corresponding band intensities of both MRP1 and Pgp1, as calculated by Image Master Software were found to increase with increasing concentration of cisplatin (Figure 1a). It reached a peak at a cisplatin concentration of 20 µg/ml, therefore the clone designated SiHa^R had been isolated at this concentration and subjected to further experiments. Level of GSH, an important determinant in drug resistance was estimated further. It was observed that in SiHa, GSH level was very high compared to normal cervical epithelial cell line (Ect1/E6E7). It was further interesting to note that in all the resistant strains of SiHa, which were developed during exposure to incremental concentrations of cisplatin, there was no change in the level of GSH (Figure 1b).

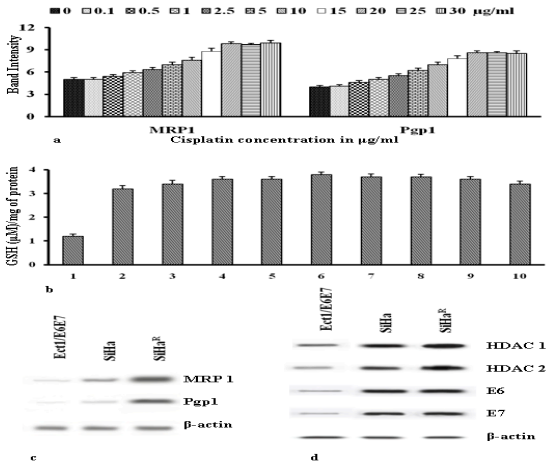

Fig. 1

Figure 1. Development of Drug Resistance and Constitutive Expressions of HDACs and HPVs in Cervical Cancer Cells. (a) SiHa cells, exposed to increasing concentration of cisplatin were subjected to western blot analysis to determine the status of MRP1 and Pgp1. Corresponding band intensities had been plotted graphically. (b) Glutathione level, an important determinant was measured at each concentration of cisplatin during development of drug resistance. 1 signifies GSH level for control Ect1/E6E7cell, 2 corresponds to SiHa, not subjected to cisplatin exposure. 3-10 corresponds to GSH level in SiHa cells exposed to 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 µg/ml cisplatin. (c) Comparison of MRP1 and Pgp1 status in control cervical cells, SiHa and SiHa^R (isolated at 20 µg/ml cisplatin concentration) using western blot analysis. β-actin was used as a loading control. (d) Constitutive expression of HDAC1, HDAC2, E6 and E7 proteins in Ect1/E6E7, SiHa and SiHa^R cells. Proteins were isolated and subjected to western blot analysis using specific antibodies. β-actin was used as a loading control

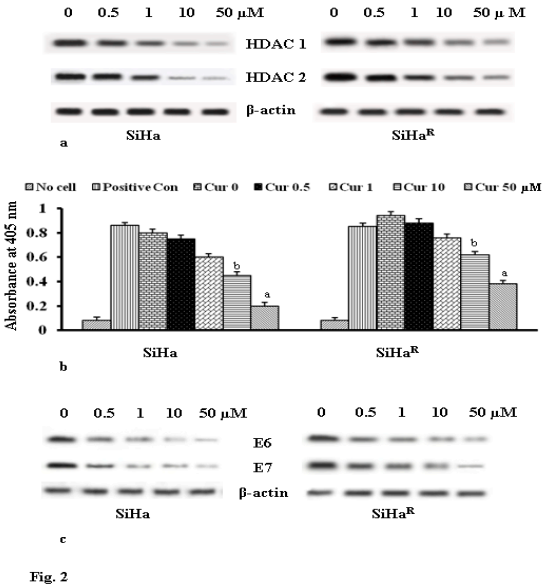

Fig. 2

Figure 2. Modulation of HDAC1, HDAC2, E6 and E7 in Cervical Cancer Cells by Curcumin. (a) Western blot analysis revealed expression of HDACs modulated by different concentrations of curcumin for 24 h. β-actin was used to ensure equal loading of protein. (b) Total HDAC activity in SiHa and SiHa^R cells after treatment with different concentrations of curcumin (0, 0.5, 1, 10, 50 μM) had been plotted. Cell extracts were incubated at room temperature with 1 mM HDAC assay substrate and reaction was terminated after 60 m`in with HDAC activator solution and finally absorbance was measured at 405 nm. No cell corresponds to the reading with assay buffer and positive control (HeLa nuclear extract) had been supplied in the kit. Results obtained were mean±SE (n=3). $^a p<0.001$ and $^b p<0.01$ represent significant changes compared to control. (c) Expression of E6 and E7 proteins had been estimated by western blot analysis with lysates of SiHa and SiHa^R cells treated with different concentrations of curcumin. To equalise protein loading, membrane was blotted with anti β-actin antibody

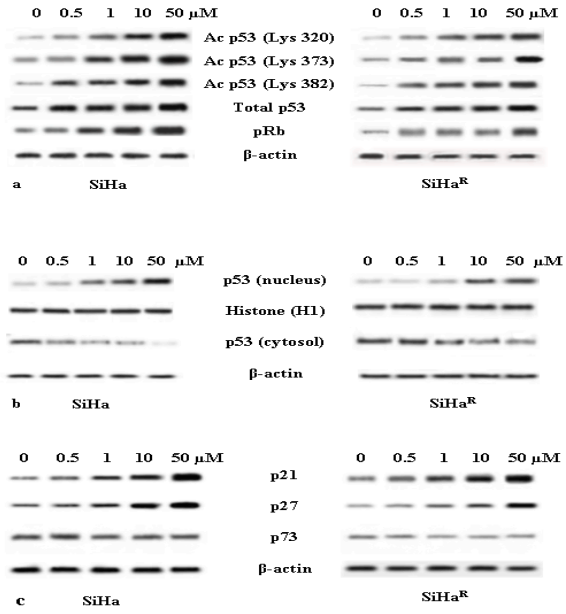

Fig. 3

Figure 3. Effect of Curcumin on Cell Cycle Regulatory Proteins in SiHa and SiHa^R cells. (a) Cells were treated with curcumin for 24 h. Expressions of acetylated p53 (Lys 320, 373, 382), total p53 and pRb was determined by western blot analysis using specific antibodies. β-actin was used as loading control. (b) Dose dependent nuclear translocation of p53 from cytosol in curcumin treated SiHa and SiHa^R cells were determined by western blot analysis. Equal protein loading in nuclear fraction was confirmed with anti-histone1 (anti-H1) antibody and that in cytosolic fraction by anti-β-actin antibody. (c) Bands obtained from western blot analysis of curcumin treated cells using antibodies against p21, p27, p73 had been shown. β-actin was used as control to ensure equal loading of protein

This result confirmed that the resistance developed in SiHa^R was not because of GSH level, but due to increase in the expressions of MRP1 and Pgp1. In Ect1/E6E7, expressions of MRP1 and Pgp1 were very poor (Figure 1c).

Constitutive expression of HDACs (1 and 2) as revealed from western blot analysis was high in SiHa and SiHa^R compared to normal Ect1/E6E7 (Figure 1d). Expression of both the HDACs was more, particularly in drug resistant clone SiHa^R compared to parental SiHa. E6 and E7 oncoprotein expressions on the other hand were high in both the cervical cancer cells lines, but there was no differential expression between SiHa and SiHa^R (Figure 1d). However, expression of these viral oncoproteins in Ect1/E6E7 was poor.

Effect of curcumin on HDACs and E6/E7 oncoproteins

Effect of curcumin on the HDAC 1 and 2 was examined in SiHa and SiHa^R cells by western blot analysis. It was apparent from the result that high expressions of HDAC 1 and 2 were observed in untreated cells, which get down-regulated with increasing concentrations of curcumin (Figure 2a). Maximum effect was observed at 50 μM curcumin. Enzymatic activity of HDAC was measured using HDAC activity assay kit. Concentration dependent inhibition of activity in curcumin treated SiHa and SiHa^R cells compared to untreated-control cells had been revealed (Figure 2b). In order to address the question whether inhibition of HDACs was due to down-regulation of endogenous HPV16-E6 and HPV16-E7, effect of curcumin on these two oncoproteins was determined by western blot analysis. E6 and E7 protein levels were decreased progressively with increasing curcumin concentration in both SiHa and SiHa^R cells (Figure 2c). This finding might support the fact that inhibition of viral oncoproteins probably modulated HDACs in cervical cancer cells.

Effect of curcumin on p53, pRb and other cell cycle regulatory proteins

HDACs control transcriptional machineries of cells by regulating acetylation of the histones as well as some non-histone proteins like transcription factors such as p53. High risk E6 proteins on the other hand bind and inactivate p53 function. Since curcumin is inhibiting HDACs (1 and 2) and E6 expression, therefore expression of p53 (acetylated and total) was examined in both the cells. Poor expression of acetylated and total p53 was observed in untreated cells (Figure 3a). Curcumin induced acetylation of p53 (Lys320, 373, 382) which was accompanied by induction of total p53 (Figure 3a). Increased acetylation

and expression of p53 thus indicated stabilization of this protein, which was otherwise either gets transcriptionally repressed due to HDACs or degraded due to viral E6 oncoprotein. pRb protein being the target of high-risk E7 oncoprotein was assessed in both the cell lines. Both of the cervical cancer cells (SiHa, SiHa^R) expressed pRb at low levels which showed a dose dependent increase in expression following curcumin treatment (Figure 3a). To understand the mechanism by which curcumin stabilized and induced p53, western blot analysis of the sub-cellular fractions were done. Results indicated that curcumin facilitated translocation of p53 from cytosol to nucleus (Figure 3b). Action of curcumin on p21 and p27 was investigated in cervical cancer cells. Up-regulation of p21 and p27 in curcumin treated cells (SiHa and SiHa^R) was observed (Figure 3c). p73, a member of the p53 gene family, however was not affected (Figure 3c). Inhibition of HDACs by curcumin therefore led to p53 acetylation and translocation to nucleus, up-regulation of pRb, p21 and p27 expressions.

Effect of curcumin on cell cycle regulation

In order to assess the specific impact of HDAC inhibition and p53, pRb, p21, p27 up-regulation on cell cycle, flow cytometric analysis of cellular DNA content of both curcumin treated and untreated cells was carried out. Histograms of cell cycle distribution revealed a continuously increasing number of cells in the G0/G1 phase with curcumin concentrations compared to untreated cells and this was coupled with a decrease in cell population in S phase indicating cell cycle arrest at G1-S phase (Figure 4a). To understand the mechanism underlying cell growth arrest, expression of Cyclin D1 and CDK4, two important regulatory protein involved in cell cycle control at G1-S transition was examined. Results revealed inhibition in the expression of these proteins by curcumin (Figure 4b).

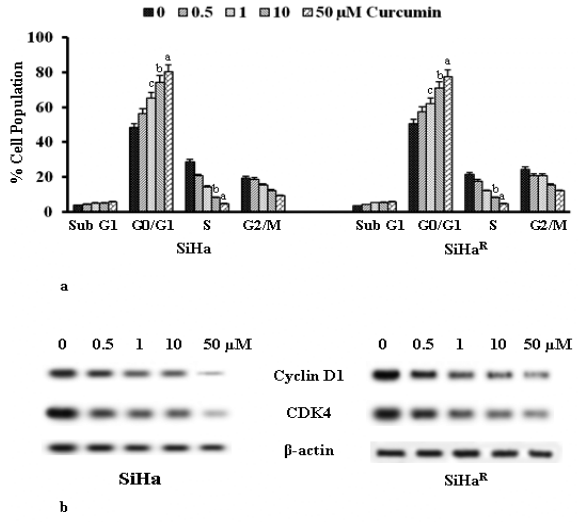

Figure 4. Curcumin Causes G1-S Phase Cell Cycle Arrest in Cervical Cancer Cells. (a) Distribution of cells treated with different concentrations of curcumin for 24 h was analyzed by flow cytometry. Histograms depicted cell cycle distribution in SiHa and SiHa^R cells. Values obtained are average of three independent experiments±SE (n=3). ^{a}p<0.001, ^{b}p<0.005 and ^{c}p<0.05 denote significant changes compared to untreated cervical cancer cells. (b) Modulation of expressions of cyclin D1 and CDK4 by western blotting. β-actin was used as loading control

Effect of modulation of MRP1 and Pgp1 by curcumin on cisplatin sensitivity

To investigate the effect of curcumin on MRP1 and Pgp1 on SiHa and SiHa^R cells, exponentially growing cells were treated with different concentrations of curcumin and the protein expression was observed by western blotting. Amount of proteins in treated and untreated cells were measured by calculating intensities of the corresponding bands using Image Master Software. MRP1 and Pgp1 expressions were very high in drug resistant strain of SiHa (SiHa^R) compared to parental SiHa (Figure 5a). Curcumin efficiently inhibited the expression of these proteins in a concentration dependent manner in both the cell lines, although extent of inhibition varied depending on the cell type (Figure 5a).

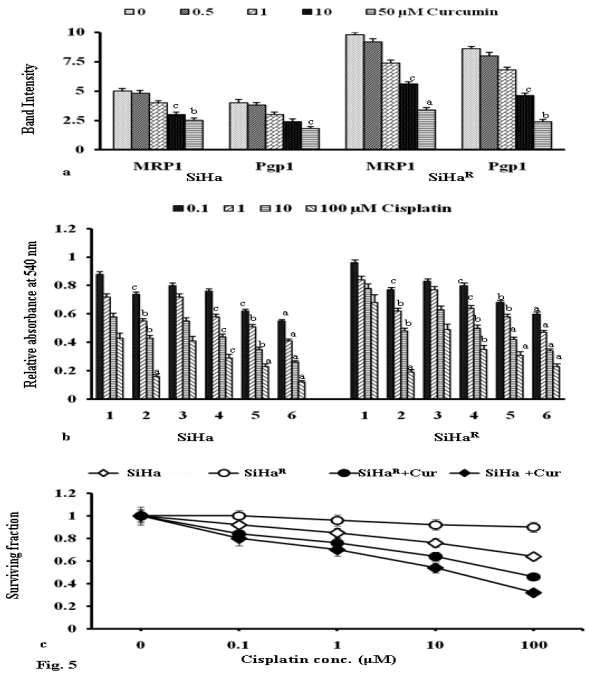

Figure 5. Regulation of MRP1 and Pgp1 Expression by Curcumin and Sensitivity of Cells Towards Cisplatin. (a) Band intensities obtained from western blot analysis had been calculated by Image Master Software and represented as bar diagram. Values are average of three independent experiments±SE (n=3). Values are significant ^{a}p<0.001, ^{b}p<0.005, ^{c}p<0.05 compared to untreated SiHa and SiHa^R cells. (b) Cytotoxicity by MTT reduction assay was carried out in cells treated with curcumin along with 0.1, 1, 10 and 100 μM of cisplatin for 24h. Bar diagram #1 represents cytotoxicity of cisplatin alone and #2 represents the same in presence of TSA (1 μM), a known specific inhibitor of HDAC. #s 3-6 represent cytotoxicity of cisplatin as modulated by 0.5, 1.0, 10.0 and 50 μM curcumin. The error bars are standard deviations of three samples. Values are average of three independent experiments. ^{a}p<0.001, ^{b}p<0.005, and ^{c}p<0.05 denote significant changes in comparison to control. (c) Surviving fraction against cisplatin dose had been plotted. Clonogenic survival assay shows that SiHa^R cells were more resistant to cisplatin than SiHa, however, presence of curcumin could help overcome the resistance of SiHa^R towards cisplatin

Based on the above findings it was furthermore investigated whether curcumin by modulating HDACs (1 and 2), E6/E7, and their target proteins and by down-regulating MRP1 and Pgp1 could enhance chemosensitivity of cisplatin in SiHa and SiHa^R. Cervical cancer cells were therefore treated with different concentrations of curcumin in combination with cisplatin (0.1, 1, 10 and 100 μM) for 24 h. Cells treated with 1 μM TSA (a known HDAC inhibitor) was used as positive control. Cytotoxicity was measured by MTT assay (Figure 5b). Result showed increased efficacy of cisplatin by curcumin in both the cervical cancer cells. EC_{50} values (effective concentration of drug required to achieve 50% of cell killing) and further relative efficacies were calculated and summarized in Table 1. EC_{50} of cisplatin in SiHa and SiHa^R cells were 80 and >100 μM respectively, which could be brought down to 8 and 20 μM by simultaneous treatment with curcumin. These values are comparable to that in presence of TSA. The relative efficacy of curcumin had been calculated and it was found to be 10 in SiHa and >5 in SiHa^R. These data suggested that curcumin treatment might be able to eliminate chemoresistance by sensitizing cancer cells to cisplatin *in vitro*.

SiHa^R cells derived from SiHa conferred resistance to cisplatin. Effect of curcumin (50μM) on these two cell lines were studied by clonogenic survival assay or colony forming assay. It was apparent from survival assay that SiHa^R, derived from SiHa by stepwise enhancement of the chemotherapeutic drug cisplatin, showed resistance to challenging doses of cisplatin compared to the parental SiHa cell. Percent survival decreases when both the cells were treated with curcumin in presence of cisplatin than that obtained with cisplatin alone. This manifestation is true for both the cells, indicating that the cisplatin resistant cells get sensitized to cisplatin vis a vis the parental SiHa. It was interesting to note that same extent of killing can be achieved at a much lower concentration of cisplatin (Figure 5c).

Discussion

The primary focus of this study was to investigate the mechanism underlying reversal of drug resistance in cervical cancer cells by curcumin, a dietary natural compound having anticancer activity. The present study showed that the expression of MRP1 and Pgp1, two important multidrug resistance proteins belonging to ABC transporter family were very high in cervical cell line SiHa, particularly in drug resistance clone SiHa^R derived from SiHa. GSH level however remained unaltered which indicated that the acquisition of drug resistance in SiHa^R was due to increased expression of MRP1 and Pgp1. These studies are consistent with that of Vasyl et al, where acquired drug resistance had been attributed to increased efflux of hydrophobic drugs due to Pgp over-expression (Chekhun et al., 2007).

Expression and activity of histone deacetylases 1 and 2, two important epigenetic regulators, belonging to class I HDAC family had been found to be down-regulated by curcumin in cervical cancer cell line SiHa and SiHa^R. Previous study from this laboratory reported inhibition of HDAC activity by curcumin in *in vitro* (Roy et al., 2011; Mukherjee et al., 2012) and *in vivo* model of breast cancer (Roy et al., 2011). HPV-E6 protein abrogates p53 protein in human cervical carcinoma cells. HPV-E7 oncoprotein interacts with HDAC1 and HDAC2 imparting immortalization; therefore effect of curcumin on these two viral oncoproteins had been studied. Results depicted inhibition of both E6 and E7 in SiHa and SiHa^R cells by curcumin. This finding was in agreement with the study of Luczak et al (Luczak et al., 2008) where Apicidin, a known HDAC inhibitor significantly down-regulated HPV16-E6 and E7 proteins.

Disruption of tumor suppressor protein p53 is a common event in cervical cancer and cervical cancer cases (Zhou et al., 2012). Overexpression of HDAC1 and E6 oncoprotein was reported to associate with destabilization and degradation of p53 (Luczak et al., 2008; Mizuguchi et al., 2012). The present findings depicted that curcumin, by inhibiting the expression of E6, E7 and HDACs induced acetylation of p53 at all the three lysine residues, thereby stabilizing the protein. At the same time curcumin facilitated translocation of p53 from cytosol to nucleus, where it is active; thus aiding proper functioning of the protein.

Functional impairment of pRb by E7 oncoprotein is a common event in HPV mediated cervical carcinogenesis (Subramanian and Chinnappan, 2013). In addition to pRb, some other target proteins of E7 are cyclin-dependent kinase inhibitors like p21 and p27 (Munger et al., 2001; Narisawa-Saito and Kiyono, 2007). Cell cycle progression is regulated by a balance between CDKs and CDK inhibitors (Wesierska-Gadek and Krystof, 2009). The present study depicted down-regulation of pRb, p21 and p27 expressions by curcumin, which coincided with the inhibition of progression of cells at G1-S phase. However curcumin failed to exert its effect on p73, another tumor suppressor protein belonging to p53 family. Cyclin D1 and CDK4, member of the cyclin family plays an important role in G1-S transition (Malumbres and Barbacid, 2009) CDKs are known to phosphorylate pRb, releasing E2F from the cytoplasm, therefore effect of curcumin on CDKs was observed. Down-regulation of two cell cycle regulatory proteins indicated that curcumin mediated inhibition of G1-S phase cell cycle arrest in cervical cancer cells was due to inhibition of Cyclin D1 and CDK4.

Effect of curcumin on multi drug resistance associated proteins was also observed. Despite considerable advances in anticancer drug development, major fundamental obstacle associated with cancer chemotherapy is development of chemo-resistance (Song et al., 2012). Among several intrinsic cellular mechanism associated with the development of multirug resistance (MDR), overexpression of plasma membrane glycoprotein 1 or Pgp1 is considered as one of the most important known mediator of MDR (Szakacs et al., 2006). Pgp1, a member of the ABC (ATP binding cassette) transporter family acts as an energy-dependent cancer drug efflux pump, prevent adequate intracellular accumulation of a large number of cytotoxic drugs (Szakacs et al., 2006). MRP1 or multidrug resistance associated protein1 is a vital protein which mediates the transport of partially detoxified compounds

such as glutathione and glucuronide conjugates (Mahjoubi and Akbari 2013).

In the present study curcumin was found to down-regulate the expressions of MRP1 and Pgp1 not only in SiHa but also in its drug resistant counterpart SiHa^R. Both SiHa and cisplatin resistant SiHa^R showed increased sensitivity to cisplatin by curcumin as evident from clonogenic survival assay. A role of curcumin in reversal of resistance to cisplatin in cervical cancer cells was observed. This might be due to suppression of MRP1 and Pgp1 expressions in SiHa and SiHa^R. Chemoenhancing ability of SiHa and SiHa^R towards cisplatin by curcumin was further confirmed by cytotoxicity assay (MTT assay). Results showed that concentrations of curcumin, which modulated E6, E7, HDACs, their downstream effector target proteins and multi drug resistance proteins, also enhanced chemosensitizing efficacy of cisplatin in SiHa and SiHa^R cells. Both the results indicated that curcumin in conjunction with cisplatin resulted into higher sensitivity towards chemotherapeutic drug. Curcumin induced down regulation of MDR genes were earlier observed by Choi et al in mouse leukemia cell line (Choi et al., 2008). HDAC inhibitors were reported earlier to down-regulate MRP2 gene expression contributing to paclitaxel induced growth arrest in MDR cancer cells (Kim et al., 2011). The present findings established curcumin as a chemo-enhancer by inhibition of HDAC and MDR proteins.

Platinum based chemotherapeutic drug cisplatin is considered as one of the most effective anticancer agents in the treatment of cervical cancer. However, severe toxicity and acquired drug resistance after therapy are the major problem of therapy. Plant derived natural compounds were reported to act as chemosensitizer due to their ability to regulate multiple survival pathways without inducing toxicity (Vinod et al., 2013). Phytochemicals have been used nowadays as an adjuvant therapy to overcome the limitations of cisplatin treatment (Tyagi et al., 2004; Ermakova et al., 2006; Sarkar and Li, 2006). Isoflavonoid genistein was reported to potentiate the sensitivity of cisplatin in cervical cancer cells HeLa (Sahin et al., 2012).

The present study demonstrated that curcumin through inhibition of HDACs, HPV16 E6 and E7 oncoproteins, positively influenced the expression of CDK inhibitor proteins with a concomitant down-regulation of CDKs, leading to cell cycle arrest at G1-S phase. The study furthermore demonstrated that suppression of MRP1 and Pgp1 by curcumin aided in enhancing the efficacy of cisplatin in cervical cancer cells. These results warranted usefulness of curcumin in reversal of resistance to chemotherapy by modulating key regulators of chemoresistance.

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