DNA damage and Cell Cycle Dysregulation by ATM-P53-GADD45A Pathway in Bleomycin Treated Cervical Cancer Cells

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Cervical cancer, DNA damage, Cell cycle arrest, Apoptosis, Cell Cycle inhibitor

ABSTRACT

Background
Cervical cancer is one of the most prevalent malignancies in women worldwide, yet the underlying mechanism of disease progression still remains unclear. The role of TGF-β1 and GADD45A and its implication in cervical cancer remains elusive. The present study aimed to elucidate the regulatory mechanisms induced by bleomycin in SiHa cells, to identify therapeutics target of DDR pathway.

Methods
In cervical cancer SiHa cells, different assays were employed to quantify the viability, anti-proliferative and anti-migratory effect of bleomycin. Differential gene expression related to cell cycle, DNA damage response, apoptosis was done by qRT-PCR with bleomycin intervention. Propidium iodide staining was done to confirm DNA damage. The relation of protein interaction with GADD45A was done by protein-protein interaction network.

Results Our results emphasises that, bleomycin treatment of SiHa cells significantly inhibits cell proliferation, migration and invasion. Downregulation of CDK1, Cyclin A and Cyclin B genes, induced cell cycle arrest at G_2/M phase, confirmed by qRT-PCR and flowcytometry analysis. Significant upregulation of p53, ATM and GADD45A related to DDR and BAX/BCL2 for apoptosis, was confirmed by qRT-PCR. Propidium iodide staining validated the chromatin fragmentation and apoptosis in SiHa cells treated with bleomycin.

Conclusion
We emphasize that in cervical cancer, bleomycin can be a potent antitumor drug targeting multiple pathways inducing cell cycle arrest, DDR and apoptosis.

1. Introduction:

The second most prevalent malignant tumour in women is cervical cancer, which seriously threatens female's health worldwide. One of the important risk factors in contributing to the development of cervical cancer is the human papillomaviruses, which is linked to cervical carcinomas both globally and Indian women [1]. In the year 2020, approximately 604,127 women were reported, and 341,831 women died with cervical cancer worldwide [2]. The process of HPV immortalization and host cell transformation depends on the E6 and E7 genes [3]. These proteins trigger the revisited hallmarks of the cancer, including induction of replicative immortality, cellular energetics dysregulation, evasion of suppressors, persistent and selective proliferative advantage, metabolic reprogramming. E6/E7 viral proteins block cyclin dependent kinase (CDK) inhibitors (p21, p27, and p16) and degrade p53 and Rb, hence impairing cell cycle checkpoint control in HPV-related cancer [4]. Other microbial infections, such as *Escherichia coli* [5], *Neisseria gonorrhoea* [6], and *Mycoplasma* [7] contribute as risk factors for cervical cancer.

Multiple treatment modalities are available for cervical cancer which include surgery, chemotherapy and radiation. Besides, combinatorial therapy is effective including surgery followed by

chemoradiation therapy. Recently targeted therapy gained importance that included PARP inhibitors [8] and bevacizumab as an angiogenesis inhibitor [9], gefitinib and erlotinib as EGFR inhibitors were extensively used to treat advanced cervical cancer [10]. As inflammation is also one of the main risk factors in cervical cancer aspirin and ibuprofen were used as nonsteroidal anti-inflammatory drugs (NSAIDs) [11]. Despite the advancement of treatment strategies 35% of the patients undergoing treatment develop recurrent or metastatic disease. Natural products that have anti-tumor properties were tried which exhibited fewer side effects though did not bring any respite to the morbidity. As HPV and other microbial infections promoted cervical cancer, we selected bleomycin, which is classified as an antibiotic and antineoplastic medication, to examine its impact on cell cycle-regulated pathways. Bleomycin is a glycopeptide that is originally isolated from *Streptomyces verticillus* and has been widely used as a chemotherapeutic drug for treatment of several malignancies, such as squamous head and neck cancers, testicular carcinomas, lymphomas, esophageal cancers, malignant lymphomas, and choriocarcinomas, due to its remarkable anticancer activity [12][13].

Extensive studies conducted on human cancers, animal models, and cell cultures have consistently shown that the accumulation of DNA damage and a deterioration in DNA damage repair pathways, both contribute to the initiation and progression of cancer, though provide targets and opportunities for cancer therapies [14]. Bleomycin ability to induce single- and double-strand DNA breaks is thought to be attributed its cytotoxicity [13]. It significantly impacts several signal transduction pathways which involved in carcinogenesis. These comprises PPAR, p53, MAPK, PI3K-Akt, and other pathways [15]. These result alters proliferation, DNA damage response, cell cycle, angiogenesis, evading apoptosis, differentiation, and insensitivity to anti-growth signals, and also affect caspases in apoptotic pathways leading to cell cycle arrest, apoptosis, and mitotic cell death. Treatment of cells with chemicals that damage DNA, such as bleomycin is linked to the activation of p53. Subsequently, the active p53 can induce apoptosis by controlling the BCL-2 protein family [16]. Tumour suppressor p53 gene is the most often mutated gene in most cancers which is crucial in maintaining genomic integrity by regulating cell cycle checkpoints and apoptotic process after exposed to genotoxic stress [17]. Activated p53 transcriptionally upregulates its downstream genes, including p21Waf1/Cip1, GADD45A, and Bax which further controlled the apoptosis and cell cycle arrest [18][19]. Furthermore, numerous studies have demonstrated that a range of upstream kinases, including ATM, ATR, p38, and c-Jun N terminal kinase (JNK) are responsible for phosphorylating p53 [19][20][21]. Induction of GADD45A in a range of mammalian cells has been seen using specific DNA damage agents. In most of the *in vitro* studies, UV treatments led to swift induction of GADD45A [22].

Thus, in this study, we underscore for the first time the role of GADD45A with the effect of bleomycin and proposed that targeting DNA repair genes could be an effective strategy for better treatment of cervical cancer. Therefore, an urgent need to elucidate the effects and mechanisms of GADD45A in cervical cancer SiHa cell line. We determined the cytotoxicity effect of bleomycin on SiHa cells and measured DNA damage and apoptotic pathway markers, such as ATM, GADD45A, p53, Bax, and BCL-2 levels in SiHa cells. Furthermore, we demonstrate that bleomycin induced DNA damage response genes by activation of GADD45A via p53 pathway and induced apoptosis.

2. Methodology

2.1 Cell culture

Cervical cancer cells SiHa (HPV 16+ve) were obtained from the National Centre for Cell Sciences (NCCS) Pune, India in the 19th passage (p19) and was tested and compared with ATCC STR profile database which showed 100% match. They were grown in DMEM medium supplemented with 10% FBS (Gibco), 100 IU/mL penicillin and 10µg/mL streptomycin, 1% glutamax, 1% sodium pyruvate, 1% non-essential amino acids (HiMedia). Cells were maintained and grown to confluency at 37 °C

in a 5% CO_2 humidified atmosphere (Thermo Scientific Forma CO_2 Incubator).

2.2 Morphology of cells

The morphological assessment of the SiHa cells after treatment with bleomycin were analysed microscopically. SiHa cells were seeded onto 6 well plate and bleomycin treatment was given for 24 h. Cells were imaged under 200X magnification using a phase-contrast microscope (PrimoVert-415510-1101-000, Carl Ziess).

2.3 MTT assay

The viability of the cells was measured using colorimetric 3-(4,5) dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate at a density of 4500 cells/well and incubated for 24 h in DMEM medium supplemented with 10% FBS. The cells were treated with different concentrations of bleomycin (0-250µM) for 24 h. Following incubation, 0.5 mg/mL MTT (HiMedia) was added into each well and incubated for 3 h. DMSO was added to each well and a microplate reader (ScanIt) was used to measure the absorbance at 575 nm. The average absorbance values of the cells were compared to the control cells to calculate the percentage of the viability and regression value from the graph to calculate IC_{50} values of bleomycin.

2.4 Cell cycle analysis

Cells treated with bleomycin were analyzed by flow cytometry for Cell cycle analysis. The treated cells were washed with sterile PBS and then harvested using 0.25% Trypsin EDTA and suspended in 10% DMEM medium. Cells were then washed with PBS and centrifuged at 1200 rpm at 4°C for 5 min and the cells were fixed in freshly prepared 75% cold ethanol and stored at 4˚C. Fixed cells were spun down, washed and then incubated with 20 mg/mL of RNase and 5µg/mL of PI in PBS for 15 min in the dark. Data from 5000 cells were collected for each sample. Data acquisition and analysis were performed on a flow cytometer (Guava Easy Cyte).

2.5 Scratch assay

Changes in migration of cells upon treatment were studied using *in vitro* scratch assay (Liang *et al.,* 2007). SiHa cells were grown to a 100% confluent monolayer in a 6-well plate, a scratch was made vertically on the monolayer by using a 200µL pipette tip. Cells were washed with sterile PBS to remove cell debris, and a fresh medium containing IC_{50} concentration of the bleomycin was added. Images of the scratch were taken at different time points, such as 0, 24, 48, 72 h, using an inverted phase contrast microscope (PrimoVert, Carl Ziess) at 4X magnification. The migration distance was calculated using ImageJ software (version 1.52a) (National Institutes of Health, Bethesda, MD, USA).

2.6 Clonogenic assay

Clonogenic assay was performed to detect the antiproliferative effect of bleomycin. Cervical cancer SiHa cells were seeded at a density of 1000 cells/well onto 6 well plate for 15 days and giving the change with fresh media treated with or without drug, once in three days. Then colonies were stained with 0.1% crystal violet stain for 15 min, excess stain was washed with PBS and number of colonies was counted using ImageJ software (version 1.52a).

2.7 Gene expression analysis

To determine the gene expression level of SiHa cells treated with bleomycin, cells were seeded in 6 well plate and cultured in 10% FBS medium at 37°C for 24 h. Further RNA extraction was done using Nucleospin RNA extraction kit method. Extracted RNA was quantified using a microvolume spectrometer (Colibri, Germany). RNA was converted into cDNA using a Takara cDNA synthesis kit. The qRT-PCR (Bio-Rad) reaction was prepared using Takara SYBR Green master mix. The qRT-

PCR was performed in BIORAD, CFX96 optics module and the reaction conditions were as follows: initial step at 95°C for 3 min, denaturation at 95°C for 10 s, followed by annealing at 60°C for 30 s, and extension for 20 s at 72°C, for a total of 45 cycles. Gene expression was normalized with GAPDH. Data analysis was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8 PI staining

Using propidium iodide (PI) staining, the impact of bleomycin on the morphological characteristics of SiHa cells was investigated. After being seeded at a density of $4x10^4$ cells/well in a 24-well plate, SiHa cells were treated with bleomycin for 24 h. Propidium iodide (1 mg/mL) was used to stained cells for 15 minutes at 37º C in the dark. To get rid of extra stain, cells were washed in PBS after staining. Cells were overloaded with PBS and using a fluorescence imager (ZOE, Bio-Rad) set up under the red channels, the cells were assessed for nuclear damage.

2.9 Protein-Protein Interaction (PPI) analysis

To better understand the dynamic process of a DNA damage and apoptosis process, information about the functional genes is required. Protein-protein interaction analysis was performed to screen important candidate genes regulated by GADD45A in cervical cancer cells using STRING database (https://string-db.org/). Experimentally validated, text mining, databases, co-expression, neighbourhood, gene fusion and co-occurrence interactions were considered for construction. In the PPI network, genes served as the nodes and edges represented the associated interactions. The connectivity degree of each node, which indicates number of interactions of the corresponding gene. Using these methods, eleven key genes, CYCA, CYCB, CDK1, TP53, ATM, Bax, BCL-2, GDF11, TGF-β and p21 association were checked with GADD45A.

2.10 Statistical analysis

All experiment was performed in triplicates and mean values are represented either as Mean ± SD or Mean ± SEM. Statistical analyses were done using students t-test and ANOVA with GraphPad prism software (version 8.01; Hearne Scientific, Pty Ltd., Melbourne, Australia). P values < 0.05 considered as statistically significant.

3. Results

3.1 Bleomycin induced cytotoxicity and morphological changes in SiHa cells

The inhibitory effects of bleomycin on SiHa cell line shown in Fig. 1 (A). The cells were exposed to different doses of bleomycin at a concentration of 0-250µM for 24 h. The relative cell proliferation progressively decreased in a dose-dependent manner. The IC_{50} value was calculated as 152µM and same concentration was used for further experiments. Morphology of the cells treated with bleomycin was assessed using an inverted phase-contrast microscope. The morphological changes were observed in the cells after treatment with bleomycin. Cells without treatment displayed normal structure and spindle-like morphology whereas, the treated cells displayed apoptotic characteristics, cell membrane disruption, dead cells and necrotic cells (Fig. 1 B, C).

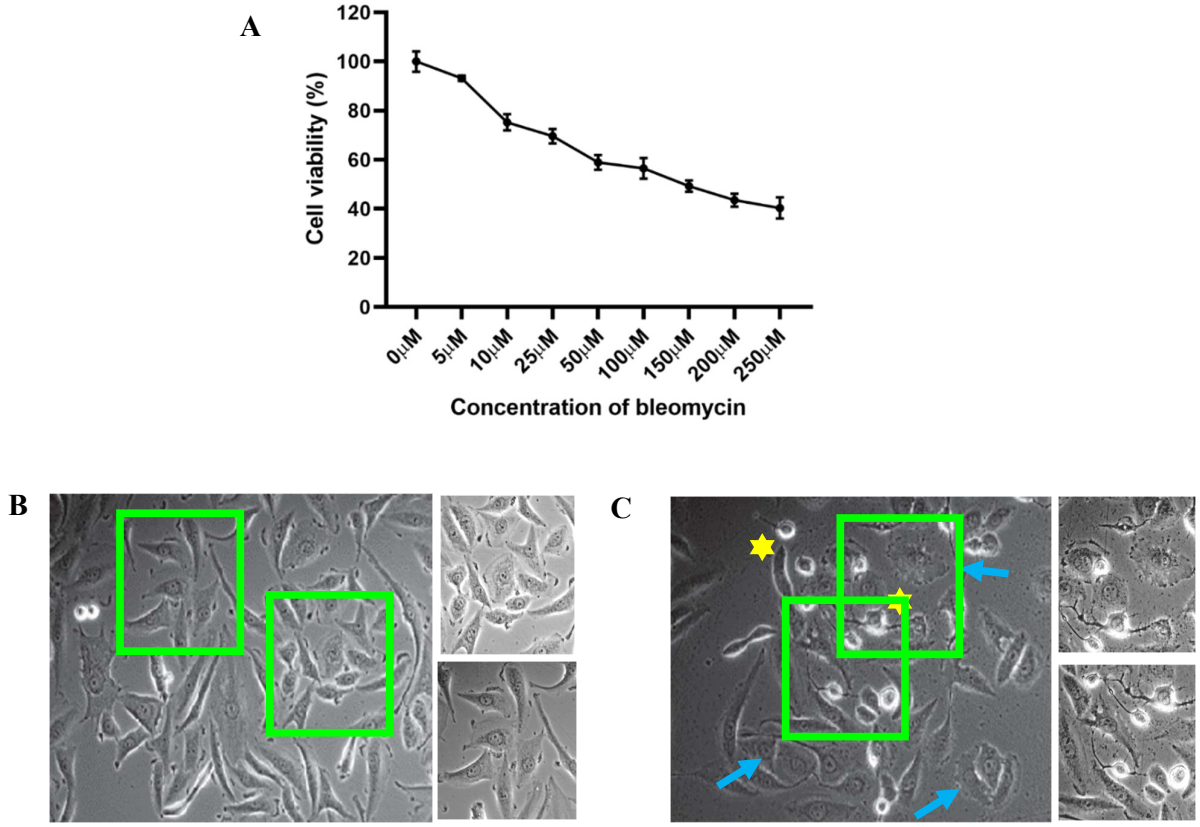
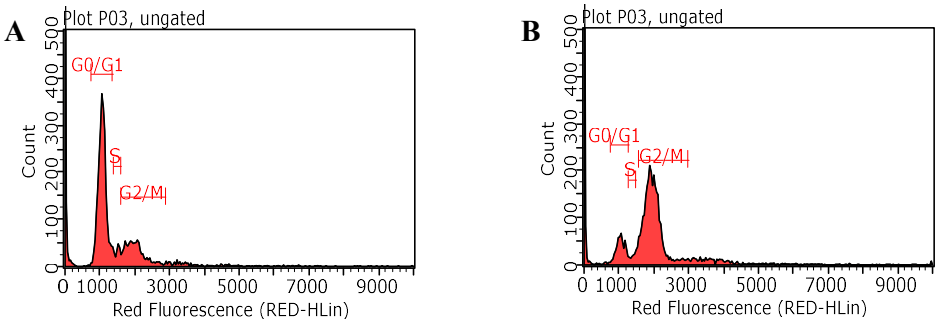


Fig. 1 Cell viability of SiHa cells treated with bleomycin. A) Cytotoxicity assay of bleomycin in SiHa cells by MTT assay. Data are presented as mean ± SD. B, C) Morphological changes in SiHa cells imaged by phase contrast microscopy after treatment of bleomycin (0 and 152µM) for 24 h. Cells showed numerous morphological changes in B (152µM). *Scale bar* = 100 µm. (blue arrows represent enlarged nuclei and cells, and yellow stars represent apoptotic cells and dead cells).

3.2 Cell cycle arrest at G_2/M phase

The induction of apoptotic bodies in bleomycin-treated SiHa cells was further analysed by flow-cytometric determination of DNA content. Histogram of DNA content obtained from PI-stained cells showed that the percentage of cells showed successive accumulation in G_2/M phase after treatment of bleomycin. The percentage at G_2/M phase in control was 19% which was increased to 53% at 152µM upon treatment with bleomycin (Fig. 2 A-C). This is regulated by irreversible DNA damage by the bleomycin treatment.



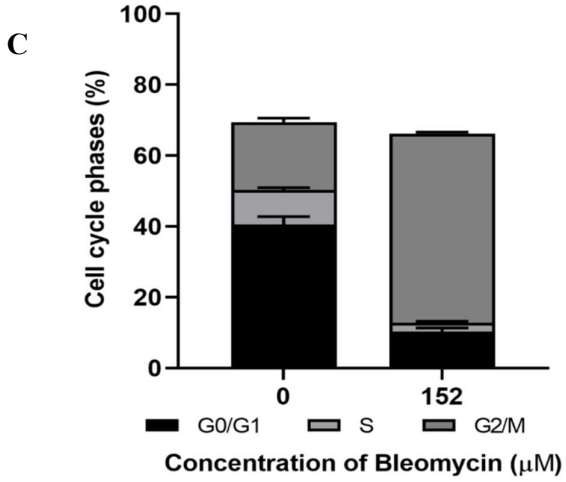


Fig. 2 Flow cytometry analysis of bleomycin-treated SiHa cells. A) Control, B) 152µM, C) Representative histogram are shown the percentage of cell at different phases of cell cycle.

3.3 Bleomycin reduces the migration capacity of SiHa cells

In vitro scratch assay is a method to evaluate the effect of drug on cell migration. Cells treated with 152µM of the bleomycin showed significant inhibition of cell migration. The gap closure rate of 152µM concentration treated cells were being assessed at 0, 24, 48 and 72 h as 14, 15, and 18 percent, respectively (Fig. 3 A, B).

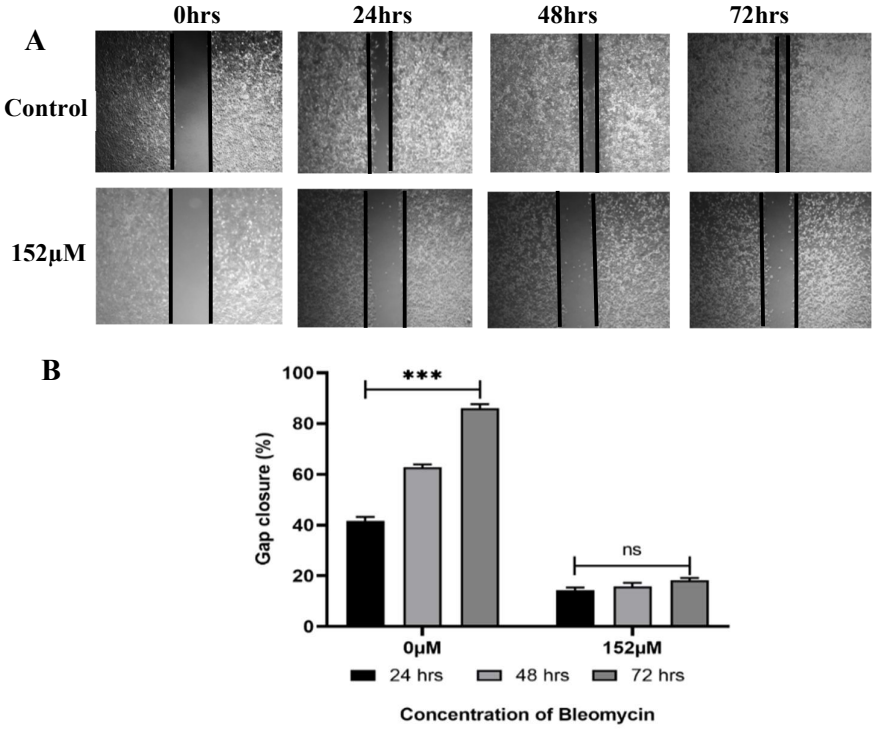


Fig. 3 The cell migration of SiHa cells treated with bleomycin. A) Images indicated area in control and treated cells. B) Bar graph showed the gap closure rate of cells at 152µM of bleomycin at different time points (24, 48, and 72hrs). (Mean ± SEM *** $p < 0.001$, ns).

3.4 Bleomycin decreases proliferation of SiHa cells

Antiproliferative effect of bleomycin on SiHa cells was done by clonogenic assay. Results showed that there was a significant difference in colony formation in non-treated and treated cells. Viability of cells was measured by counting the colonies formed and the number of colonies in control were observed as 192, whereas there is no colony formation at 152µM (Fig. 4 A, B).

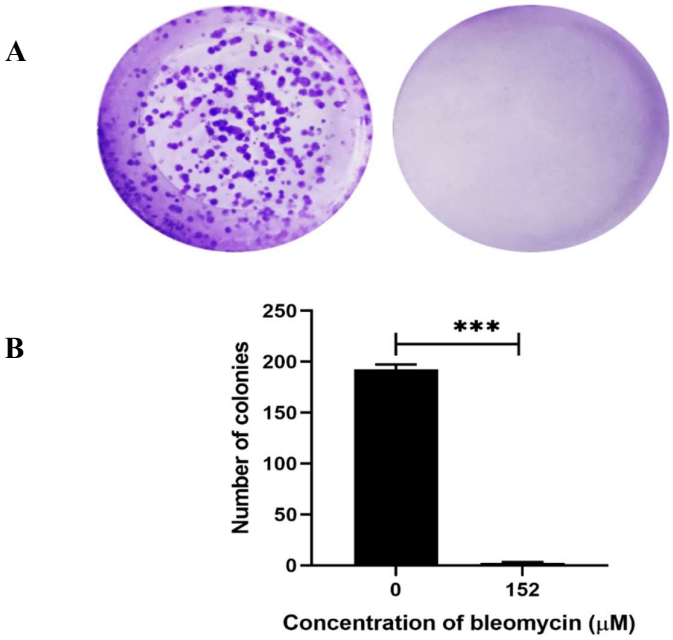


Fig. 4 The clonogenic proliferation of SiHa cells treated with bleomycin. A) Clonogenic proliferation of SiHa cells after 15 days of treatment with bleomycin. B) Histogram showed the mean number of colonies (Mean ± SEM *** $p < 0.001$).

3.5 Effect of Bleomycin on cell proliferation and cell cycle related proteins

It was interesting to investigate that how bleomycin affected cell cycle regulatory molecules since flow-cytometry data revealed the cell cycle arrest in bleomycin-treated SiHa cells. Genes responsible for cell cycle arrest were evaluated using qRT-PCR. The expression of Ki67 (Fig. 5A) is strongly associated with tumor cell proliferation and growth. There was significant decrease in the expression of Ki67, which is in response to p21 gene. Results showed that there was a significant increase in the expression of p21 (Fig. 5B), which is a cell cycle regulatory gene. The tumor suppressor gene, p53 showed the significant increase at 152µM in SiHa cells treated with bleomycin. p53 gene induced the expression of p21 in response to bleomycin treatment and induced cell cycle arrest and apoptosis, which is regulated by p53-p21 pathway or p53 dependent pathway. The results showed that there was significant decrease in the Cdk1, cyclin A, and cyclin B genes expression in the cells treated with bleomycin (Fig. 5C-E), which showed arrest at G_2/M phase of cell cycle.

3.6 Bleomycin induced DNA damage and apoptosis in SiHa cells

In order to investigate the mechanism by which bleomycin induced DNA damage and apoptosis, the expression of p53, ATM, GADD45A, Bax, BCL-2 was assessed using qRT-PCR. The results indicated that the upregulation of p53, which was in response to DNA damage. Subsequently, the activated p53 mediated apoptosis is regulated by the BCL-2 family. Data showed that there was significant increase in the expression of ATM gene. The pro-apoptotic gene i.e., Bax showed the significant increase in response to bleomycin treatment, whereas the anti-apoptotic gene, BCL-2 showed significant decrease in the expression level. Another gene responsible for DNA damage and

growth arrest is GADD45A, which is a downstream gene of p53. Results indicated that there was a significant increase in the expression of GADD45A, which is in response to p53 and DNA damage (Fig. 6 A-E).

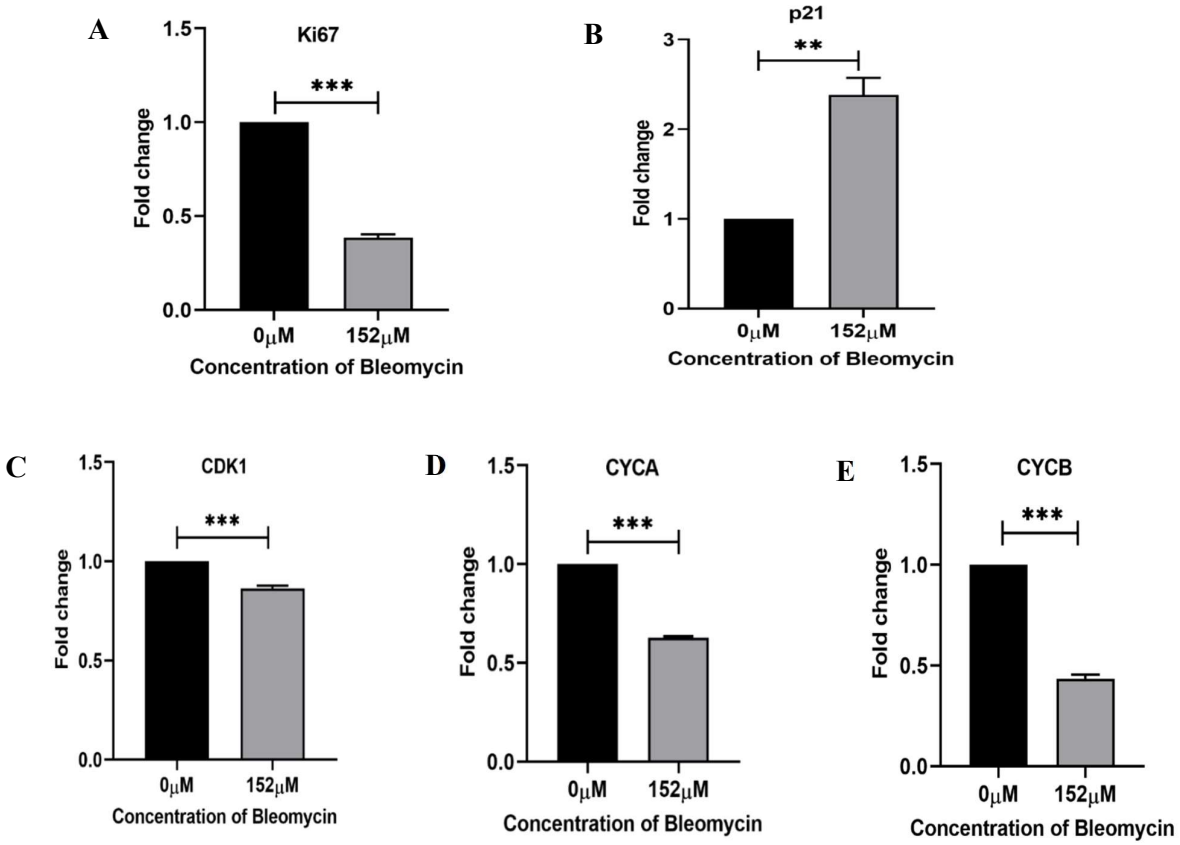
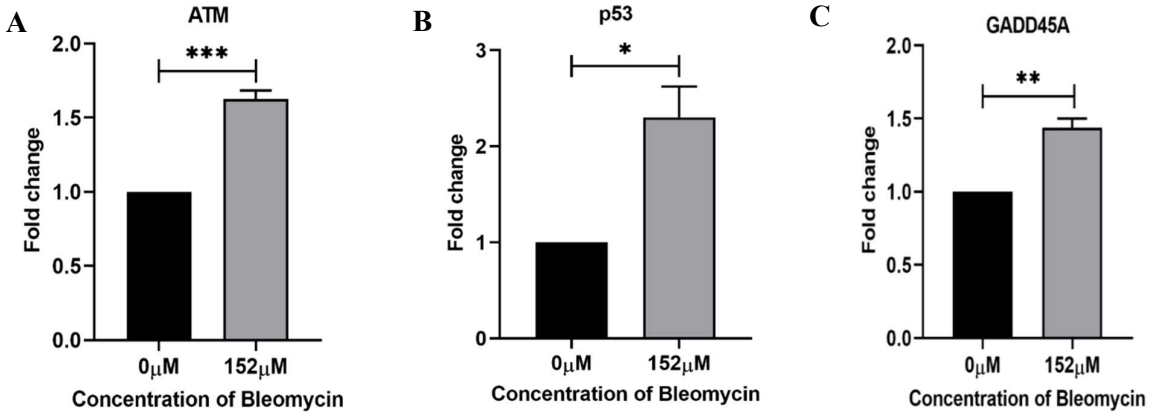


Fig. 5 Expression analysis of cell cycle regulatory genes (A) Ki67, (B) p21, (C) CDK1, (D) Cyclin A2, (E) Cyclin B1 in the SiHa cells treated with bleomycin and analysed using qRT-PCR. (Mean ± SEM, *** $p < 0.001$, ** $p < 0.01$).



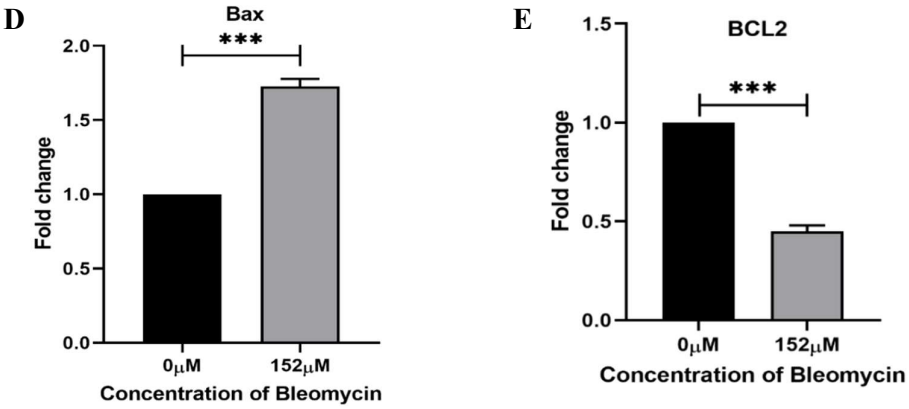


Fig. 6 Expression analysis of cell cycle regulatory genes (A) p53, (B) ATM, (C) GADD45A, (D) Bax, and (E) BCL2 in the SiHa cells treated with bleomycin and analysed using qRT-PCR. (Mean ± SEM, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$).

3.7 Bleomycin affect the downstream players and effectors of TGF-β signaling pathway

To investigate the other mechanisms by which bleomycin induced cell cycle arrest and apoptosis, the expression of TGF-β and related genes were assessed using qRT-PCR. The results indicated that the TGF-β showed upregulation upon bleomycin treatment. Other member of TGF-β superfamily gene is GDF11 was analysed by qRT-PCR. There was a significant increase in the expression of GDF11 in SiHa cells treated with bleomycin (Fig. 7 A, B). Thus, these results suggested that TGF-β and GDF11 also controlled the proliferation by inducing the expression of p21 and inhibited by CycA2/CDK2 and CycB1/Cdk1 complex upon bleomycin treatment.

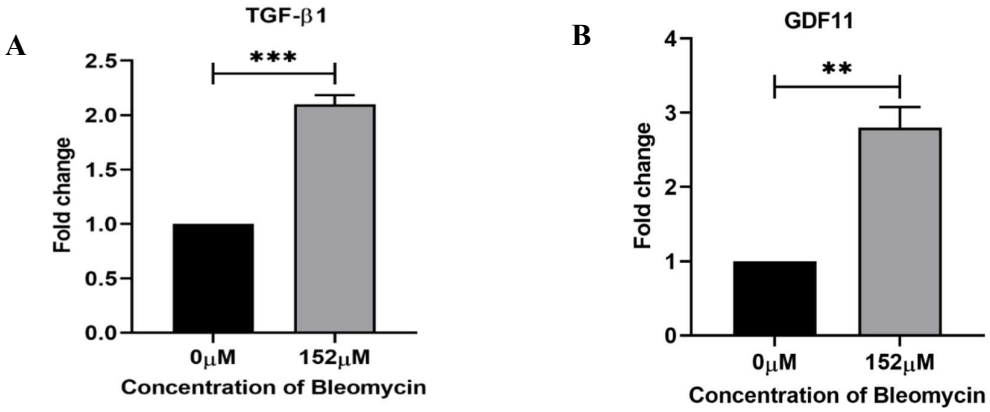


Fig. 7 Expression analysis of TGF-β family genes (A) TGF-β1, (B) GDF11 in the SiHa cells treated with bleomycin and analysed using qRT-PCR. (Mean ± SEM, *** $p < 0.001$, ** $p < 0.01$).

3.8 Bleomycin disrupts chromatin structure

Bleomycin disrupts DNA by intercalation in between the bases and induced DNA damage. PI staining was performed to comprehend the chromatin architecture upon bleomycin treatment. SiHa cells were observed under fluorescence microscopy following PI staining after treatment. The microscopic observations revealed that the bleomycin treatment caused chromatin fragmentation, nuclear shrinkage and late apoptosis in SiHa cells (Fig. 8 A, B).

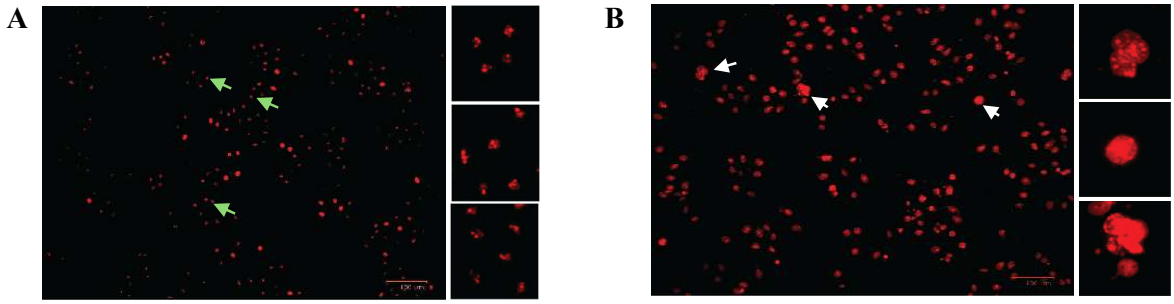


Fig. 8 Fluorescent microscopy images of the bleomycin treated and untreated cells. SiHa cells were incubated with bleomycin for 24 h. Propidium iodide-stained SiHa cells that were untreated (A), 152µM (B). The green arrow heads indicate the normal nuclei with intact chromatin. The white arrow heads points to cells with abnormal nuclei and indicate nuclei/chromatin fragmentation.

3.9 Protein-protein interaction (PPI) Network Analysis

The list of eleven common GADD45A binding partners was imported into the STRING database for further investigation. STRING analysis revealed that nearly all common GADD45A binding partners were highly connected. To calculate the interaction confidence in the network each edge is assigned a score as the edge weight. This score represents the estimated probability that a given interaction is biologically meaningful. PPI with a medium confidence score 0.4 were selected to ensure the quality of interactions. The PPI data for cell cycle regulatory genes from SiHa cell line revealed 11 nodes and 39 edges. The average node degree was 7.09 and clustering coefficient was 0.889 considered for STRING analysis. Exploration of protein connectivity showed that the seven key genes CDKN1A (p21), TP53, BCL-2 and ATM, CDK1, CCKN2B, CCNA2 were highly connected with GADD45A. Notably, other two hub genes TGF-β, Bax were connected with TP53. Whereas, GDF11 showed only one connection with TGF-β, which was based on other interactions such as protein homology, text mining and co-expression (Fig. 9).

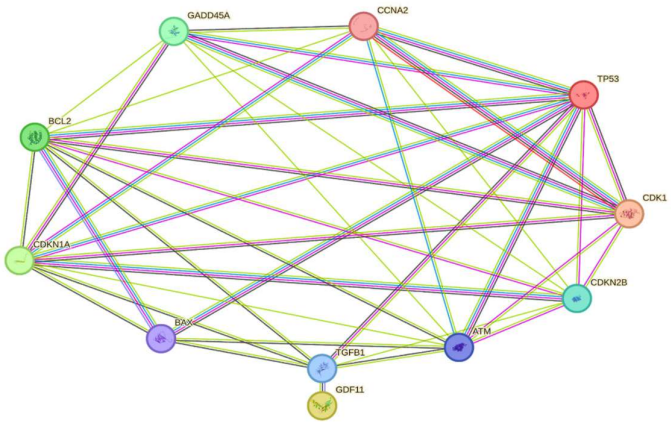


Fig. 9 Protein-protein interaction network of GADD45A binding genes. String Protein-protein interaction (PPI) network analysis of ten common GADD45A-interacting genes reveals nearly all genes are highly interconnected.

4. Discussion

Imbalance between cell proliferation and cell death leads to carcinogenesis. A sequence of cellular responses including regulators and effectors are involved in response to carcinogenic, genotoxic, and physiological stimuli. The most altered molecule in cancers is p53, which triggers the production of p21 in response to cellular stressors including oxidative stress or DNA damage [24]. It has been

shown that p21, a well-known cyclin-dependent kinase (Cdk) inhibitor, crucial in regulating the advancement of the cell cycle [25]. In addition to cell cycle arrest, p21 is also involved in senescence through both p53-dependent and p53-independent mechanisms [26]. In the present study, bleomycin induced the cell cycle arrest at the G_2/M phase exhibiting 3-fold change in the expression of p53 and p21. The upregulation of p21 further downregulated the expression of Ki67, cyclin A, cyclin B and CDK1 to 0.5 fold. This downregulation indicates the inhibition of cell proliferation and cell cycle arrest.

Bleomycin damages DNA by binding to metal ions forming metallo-bleomycin complexes which triggered reactive oxygen species (ROS) generated by these complexes causing DNA single-strand and double-strand breaks [27]. DNA double-strand breaks are the most disastrous abrasions induced by chemotherapeutics drug and can trigger a sequence of cellular DNA damage responses (DDRs). These responses include cell cycle arrest, DNA repair, and the activation of DNA damage sensing and early transduction pathways [14]. The tumor-suppressor TP53 and its downstream components have been demonstrated to play the crucial role in cellular response to a different agent that damage DNA. The first well-defined TP53 downstream gene, GADD45A instantly triggered by DNA damage caused by genotoxic drugs including 5-fluorouracil and cisplatin, as well as radiation. Furthermore, GADD45A is implicated in many growth regulation mechanisms, apoptosis, DNA damage, and cell injury [28]. Moreover, it was shown that GADD45A inhibits mTOR/STAT3 pathway to downregulate VEGF-A production, therefore suppressing tumor angiogenesis. There are numerous and intricate regulatory networks linked to GADD45A expression regulation, including BRCA1, FOXOA3, ATF4, p38, and JNK stress mitogen-activated protein kinases (MAPK) [29]. GADD45A contributes to the cell cycle checkpoint mechanism by interacting with cdc2/cyclinB1 to displaces PCNA [proliferating cell nuclear antigen) from the cyclinD1 complex, delaying the cell cycle in S phase or G_2/M phase [30]. GADD45A is believed to be a tumor suppressor of human malignancies, such as breast cancer, prostate cancer, and gastric cardiac adenocarcinoma [31].

We observed the significant increase in the expression of ATM and GADD45A in response to p53. There is 1.5-2-fold change in the bleomycin treated cells. These finding suggest that our data is in consistent with other reports that GADD45A plays an important role as tumor suppressor by inhibiting Cdk1-cyclin B1 complex in G_2/M phase of cell cycle (Fig. 10). In this study, the increased expression of GADD45A was observed, in response to DNA damage that led to increased p53 and ATM expression. ATM kinase plays a crucial role in the maintenance of genomic stability. GADD45A is involved in the ATM-p53 mediated DNA damage response and regulates cell cycle arrest, apoptosis, and DNA repair. Further the expression of apoptotic markers BAX and BCL-2 were evaluated. The ratio of BAX/BCL-2 is an important factor to induced the apoptosis. Our results showed that the BAX/BCL-2 ratio was 3.90, which was significant for apoptotic induction.

Cellular responses to DNA damage are important elements that influence the development of cancer and outcome following chemotherapy. By identifying the molecular mechanisms controlling DNA damage signalling and DNA repair in response to various kind of DNA lesions allows for a better understanding of the effect of chemotherapy on normal and tumor cells [32]. Multiple pathways serve as therapeutic targets for various compounds that include phosphatidylinositol 3-kinases (PI3K) protein kinase B (or Akt) [33], hypoxia-inducible factor-1α (HIF-1α) [34] and extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) [35], Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) [36]. Besides, transforming growth factor-β (TGF-β) signalling pathway is paradoxically pleiotropic in nature where TGF-β acts both as tumor suppressor or as tumor promoter. It plays an important role in growth, differentiation, cell death and migration [37]. Tumor promoting role is by inducing EMT through SMADs, ERK, MAPK, PI3K,

AKT, β-catenin, Ras and c-Fos. Tumor suppressor role is by activating p15, p21 and inhibition of c-MYC expression. Deregulation of TGF-β signalling plays critical role in tumorigenesis [38].

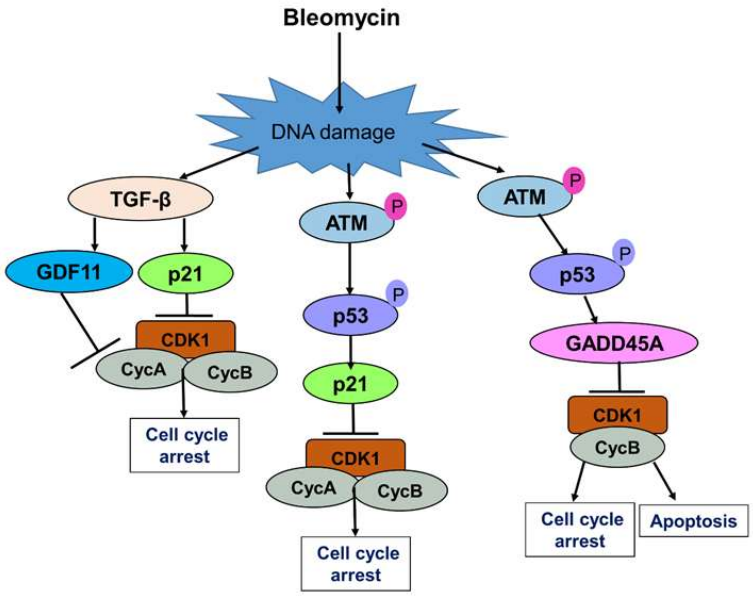


Fig. 10 Schematic diagram depicting the mechanism by which bleomycin exerts its anti-tumor effects. Bleomycin induced DNA damage triggering ATM that phosphorylates p53, further p53 binds to either p21 or GADD45A which auxiliary disrupts the Cdk1-kinase complex thus, arresting cells at G_2/M phase which leads to cell cycle arrest and apoptosis. Concurrently, bleomycin targets TGF-β mediated signalling pathway. TGF-β activates p21 and GDF-11, it leads to cell cycle arrest by inhibiting Cdk1-CycA-CycB complex.

The initiation and cooperation of several mechanisms to block cell cycle progression are critical for the complete repair of damaged genome [39]. Transforming growth factor-β (TGF-β) signalling has been shown to control the DNA damage response (DDR) [40]. TGF-β signalling inhibition reduces ATM activity, causes instability in the genome, and increases sensitivity of the cell to radiation [41]. Activation of both TGF-β signalling and DDR induces growth arrest of most cell types. DNA-dependent kinase (DNA-PK), ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), and members of the phosphatidylinositol 3-kinase (PI3K)-like kinase family initiate DDR signalling in response to DNA damage [42][32]. After ionising radiation and chemotherapy-induced double-strand breaks are detected, ATM triggers cell-cycle checkpoints. ATM kinase may phosphorylate a variety of substrates implicated in cell-cycle checkpoint in the nucleus during DDR-induced activation [42][43]. By upregulating the cyclin-dependent kinase inhibitor CDKN1A (p21), ATM-mediated phosphorylation activates and stabilises tumor suppressor p53, which in turn induces cell-cycle arrest and facilitates DNA damage-induced apoptosis [43][39]. Our results suggested that the upregulation of ATM induced the expression of TGF-β in retaliation to DNA damage response (DDR). In the present study, the expression of TGF-β and GDF11 was also evaluated, which shows the increased expression upon bleomycin treatment, in addition to p21, ATM and p53, which acted as tumor suppressor genes in cervical cancer cell line.

Conclusion

We emphasise that the DNA damage response pathways activated following bleomycin intervention that delineates the mechanisms determining role of ATM and GADD45A. The current study

underscores the role of bleomycin to inhibit proliferation, cell migration and invasion, inducing cell cycle arrest and apoptosis in SiHa cells by ATM-p53 pathway. Further, we discussed the approaches to develop the bleomycin as a therapeutic agent by modulating DDR with a goal of enhancing the effectiveness for cervical cancer therapies. By delving into the realm of DNA damage and its response, we aspire to explore more precise and effective strategies for cervical cancer therapy and to seek novel possibilities for intervention. Therefore, bleomycin can be proven as promising target for cervical cancer therapy.

Competing interest The authors have no conflicts of interest to declare.

Author contributions

SS: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing- original draft, Writing-review and editing. **RCK:** Conceptualization, Funding acquisition, Supervision, Writing-review and editing

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