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Research Article
Effect of Genistein on E-Cadherin as Biomarkers in Diagnosis and Treatment of Lung Cancer

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Abstract

Background and Objective: The prevalence of lung cancer is significant enough in recent times and the research is not able to provide availability of treatment for a late-stage cancer patient to increase the survival time and rate. Among many established markers, E-cadherin (involved in cell-cell interactions) has a crucial role to play in subsiding tumour growth and its malignancy. Therefore, the study aims to investigate the effects of Genistein on cell viability on lung cancer cell lines and its expression patterns on E-cadherin and EGFR. **Materials and Methods:** Lung cancer cell lines were grown and assayed for cell viability for Genistein compared with Cisplatin. The protein samples were further extracted with radio-immunoprecipitation assay and carried forward for western and northern blotting. Furthermore, the effect of Genistein as apoptotic cell death-inducing activity was analyzed using a flow cytometer and estimated with Caspase 3 expression. **Results:** In the presence of Genistein, cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 showed cell viability less than 40%, at varying concentrations. Genistein at concentration 0.1 μM showed cell viability less than 20% in cell lines NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322. Further western blot showed the expression of E-cadherin in all cell lines signifying them as biomarkers in NSCLC treatment. Results from flow cytometry also supported the significant effects of Genistein on NSCLC cell lines. **Conclusion:** The study proposes E-cadherin could be a biomarker for diagnosis of Non-small Cell Lung Cancer (NSCLC) and Genistein remediated up-regulation of E-cadherin could prove to be a novel outcome for the treatment of cancer and prevent malignancy.

Key words: Lung cancer, genistein, E-cadherin, EGFR, caspase, western blot, flow cytometry

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancerous mortality worldwide with lung carcinoma is reported to be very high and needs great attention to deal with it. Type lung cancer depending upon morphology and source is of two types i.e., non-small Cell Lung Cancer (NSCLC) which are large and round e.g., adenocarcinoma and Small Cell Lung Cancer (SCLC) are emerged due to change in expression of different cells (mutation)[1]. A total of 80-85% of lung cancers are NSCLC, while SCLC also known as oat cell cancer persists about 10-15% of all lung cancers. Chemotherapy for NSCLC remains marginally effective[2]. Normal lung epithelial cells with an epigenetic and genetic variation that modulates growth and finally transformed to NSCLCs[3]. Higher occurrence of NSCL Cancer increased the focused research in the field of personalized targeted therapies resulting in scope for new horizons and expectations for treatment in cancer patients[4]. Although there is advancement in early detection and standard treatment, most patients have a poor prognosis, with only a 10-15% chance of an overall 5-year survival rate. There is also a need for predictive biomarkers for the identification of tumours to implement targeted therapies as advanced treatment[5]. Epithelial cadherin (E-cadherin) a glycoprotein could be a predictive biomarker for the identification of NSCLC cancer and helps in treatment. It has three domains: A short intracellular, a transmembrane and a large extracellular domain. Coalescence of different catenins i.e., b-catenin, g-catenin and a-catenins of 88, 80 and 102 KDa, respectively with cellular actin cytoskeleton reported being controlled by E-cadherin[6]. Reports of metastases in human tumours showed E cadherin downregulation, suspecting it as a cause of metastasis. In most of the NSCLC carcinomas and their specific cell lines EGF-R was reported to be overexpressed[7]. Inverse relationships have been observed between the activation of EGF-R and the expression pattern of E-cadherin in many cell lines[8]. E-cadherin was observed to affect EGFR activation and signalling pathway through its downstream targets. E-cadherin inhibited activation of EGFR ligands, in addition, enhanced activation of protein kinase B (AKT) in neighbouring cells[9]. In NSCLC cell lines, signalling pathway of h-catenin and zinc finger proteins mainly ZEB1 (ZFHX1A, yEF1, AREB6 and TF-8), Slug/Snail family and SIP1regulated the E-cadherin expression[10]. Its regulation mechanism involved the interaction with two promoter sequences comprising 5V-CACCTG (E-box). Expression of ZEB1 and E-cadherin showed correlation with gefitinib (TKI based drug) sensitivity. Restoration of expression of E-cadherin by HDAC inhibition due to gefitinib enhanced the correlation[11]. The E-cadherin-catenin complex has been reported critical to improved intercellular adhesiveness and maintained normal tissue architecture. The down-regulation and reduction of E-cadherin resulted in tumour invasion, metastasis and unfavourable prognosis[12]. Genistein, an isoflavone extracted from soy, showed significant effects on cancer cells, including inhibition of growth and metastasis of cells, DNA methylation, enhancement of histone acetylation, inhibition of activation of NF-κB and anti-oxidant, antiangiogenic and anti-inflammatory effects[13,14].

In the present study, the effects of Genistein on cell viability on various lung cancer cell lines and the expression patterns of E-cadherin and EGFR along with apoptosis pattern with flow cytometry and caspase enzyme was studied.

MATERIALS AND METHODS

Study area: The study was carried out at the Medical Research Facility of the Wusheng People's Hospital, Wusheng County, Guang'an, Sichuan, China from July, 2019-June, 2021.

Cell culture: Cell lines of NSCLC (PC9, A549, NCI-H460, NCI-H1155, NCI-H1299, NCI-H820, NCI-H157, NCI-H838, NCI-H1666, HCC4011, HCC4006, HCC2279 and H322) were procured from American Type Culture Collection (ATCC). Penicillin, streptomycin and fetal bovine serum (10%) supplemented RPMI 1650 maintenance media was used to maintain the cell lines.

Growth assay and cell viability: Cells (5×104 cells mL^{-1}) were first plated on 96 well plates (37°C, 5% CO_2) and after 24 hrs of growth used media was discarded and 120 µL RPMI 1650 media containing serially diluted drug (Genistein) was added. About 96 well plates were placed in an incubator set at temperature 37°C and buffering with 5% carbon dioxide for 48 hrs. Cell viability study was performed after 48 hrs growth of cell culture using the reagent i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which are reduced to formazan as purple crystals by viable cells thus retained while dead cells are not able to reduce the reagent. About 20 µL MTT of concentration 2 mg mL^{-1} in saline phosphate buffer (pH 7.0), was added to each well containing 48 hrs grown cells and allowed to incubate for at least 2 hrs at 37°C. The cells were then exposed to centrifugal force $1000 \times g$ for 10 min to separate cells from liquid media and washed 2 times with saline phosphate buffer (pH 7.0) to remove the remaining media. Centrifuged cell pellets were

treated with 200 µL Dimethyl Sulfoxide (DMSO) in the well of the microtitre plate to dissolve blue-coloured formazan crystals. UV absorbance in form of optical density at 540 nm was taken for each well of cells' growth in the microtitre plate reader. Effect of Genistein was studied on the growth of cell line and results were compared with Cisplatin as a standard drug. All the experiments were performed in triplicates.

Results were represented as[15]:

$$\text{Viability (\%)} = \frac{\text{Mean OD of test wells}}{\text{Mean OD of control wells}} \times 100$$

Western and northern blots: Protein samples were extracted with Radio-immunoprecipitation Assay (RIPA) buffer (Abcam Scientific) containing phosphates inhibitors (Abcam Scientific) and protease from treated cells of different cell lines and quantified using a BCA kit (Thermo Scientific). In 8% SDS-PAGE, proteins were resolved and electrophoresis was performed and then blotting over the nitrocellulose membrane. The blotted membrane was further treated with 5% skimmed milk (in phosphate buffer solution with 0.1% tween-20, PBST) at 25°C for 60 min. Antibodies specific to EGFR (Vector, Novocastra, Burlingame, CA) and E-cadherin (Cell Signalling Technology, Danvers, MA) was diluted at 1:500, 1:1000 and 1:5000, respectively, in 5% milk in PBST buffer. PBST washing for 5 min for at least 5 times was given to the membrane anti-mouse IgG, horseradish peroxidase-linked Antibody (Cell Signalling Technology, Danvers, MA) at dilution of 1:1000 in 5% skimmed milk in PBS buffer was added to the membrane and incubated for 60 min. After incubation, the membrane was given PBST washing for 5 min for at least 5 times and the strength of the chemiluminescent signal was observed using a chemiluminescent detector as per the instruction manual (Invitrogen™ iBright™ Imagers). To perform Northern blots, 1.2% formaldehyde-agarose gel was done to separate 10.0 g of RNA using electrophoresis. Isolation of RNA (using CsCl gradient) and transfer of RNA by Northern blotting was done as per standard procedures (Pierce, Rockford, IL).

Apoptotic cell death induction study by flow cytometry: The effect of Genistein as apoptotic cell death-inducing activity was executed with a flow cytometer. Annexin V-FITC and 7-Amino-Actinomycin D (7-AAD) was taken as early apoptosis and late apoptosis detection fluorescence reagent. Forty Eight hours grown control and test cells were first made detached from the surface by treatment with trypsin proteolytic enzyme. The detached cell suspension was incubated for 20 min in binding buffer with no dilution than with annexin V-FITC (Sigma Aldrich) and 7-AAD (Thermo Fisher Scientific) as per instructions given in the supplier's user manual. The fluorescent cell suspension was presented to the flow cytometer (FlowSight®, Amnis®, Millipore) and cell sorting data were processed and analyzed.

Estimation of caspase 3 expression: Approximately 1×103 NSCLC cells were incubated for 24 hrs at 37°C, 5% CO_2 in each well of the plate. After incubation, luminescent Caspase 3 Glo solution supplied by Promega at the same volume was added into each well of 96 well plates. Mixing of sample in plates was done by shaking. Then, luminescence representing a concentration of caspase 3 was detected by a plate reader (BioTek) and changes were evaluated. Results for Genistein-treated cells compared with 1% DMSO treated cells (controls).

RESULTS

Thirteen NSCLC cell lines (PC9, A549, NCI-H157, NCI-H460, NCI-H820, NCI-H838, NCI-H1155, NCI-H1299, NCI-H1666, HCC4006, HCC4011, HCC2279 and H322) procured from ATCC were included in the present work. The effect of Genistein on the cell growth of these NSCLC cell lines was studied. Cisplatin (100 µM) was taken as a standard drug. Genistein showed a significant effect on the cell. We found a less than 40% cell viability in the case of cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 after 48 hrs (Fig. 1).

Results for cell viability using Genistein (100 µM) were equivalent to Cisplatin (100 µM) in the case of cell line A549 (cell viability: 33±1.8%) and H322 (cell viability: 25±1.7%). In the case of NCI-H820, NCI-H838 and NCI-H1155, results for cell viability using Genistein (100 µM) were similar to that of Cisplatin (100 µM) with a variation of ≤7%. Further cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 were exposed with varied Genistein concentrations ranging from 0.001-10000 µM for 48 hrs to evaluate the optimum concentration of Genistein for tumour treatment (Fig. 2).

NCI-H820 showed a steep fall in cell viability at 0.01 µM Genistein from 70-30% at 0.1 µM. While in the case of another cell line gradual decrease in cell viability was observed from 0.01-1 µM concentration except for cell line A549. It is worth noting that Genistein at low concentration showed a significant effect on NSCLC cell lines. Moreover, inverted

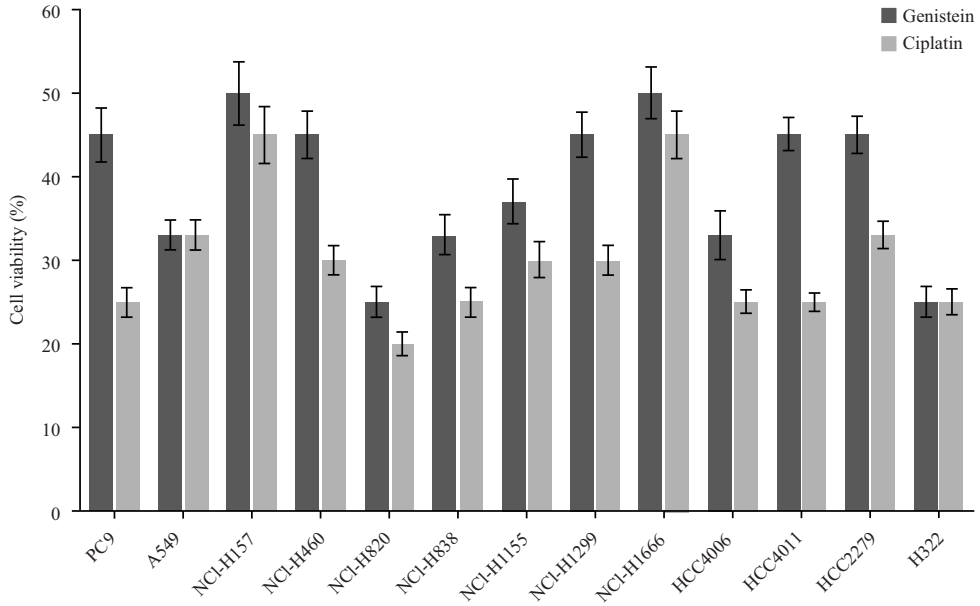

Fig. 1: Effect of genistein (100 μM) and standard drug (cisplatin (100 μM)) on cell viability of different cell lines

100 μM of genistein and cisplatin was given as treatment to cells for 48 hrs. Results are presented here as Mean±standard deviation of three parallel outcomes of each study

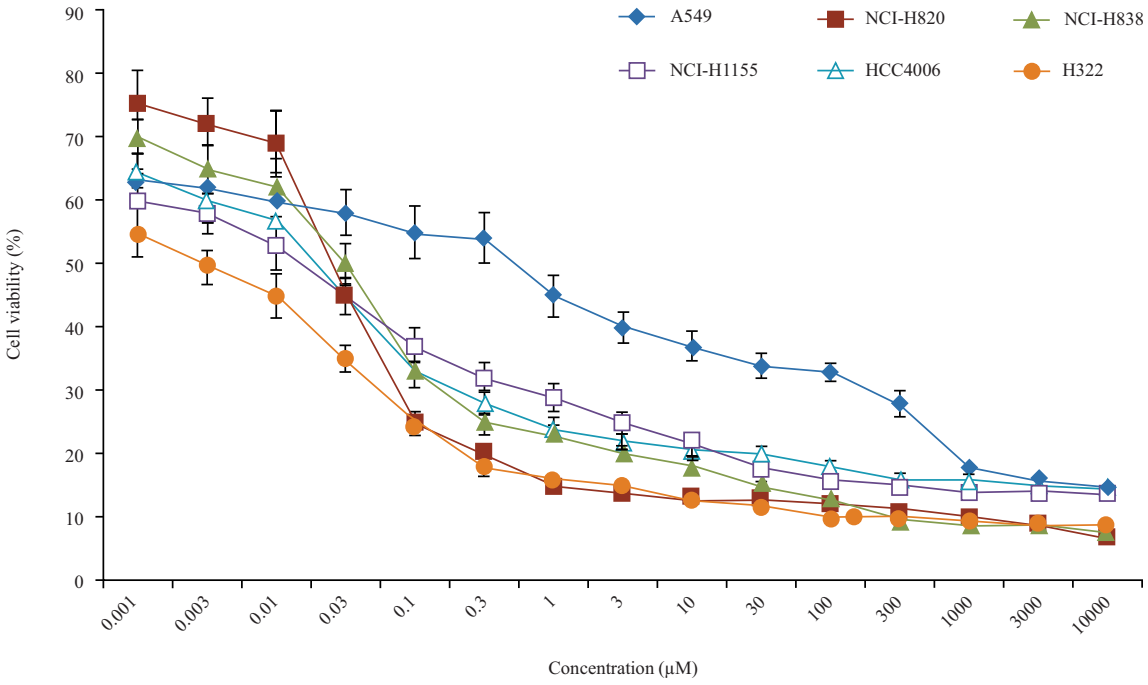

Fig. 2: Effect of genistein at the different concentrations on the viability of NSCLC cell lines (cell viability <40%)

Cells were exposed to drugs ranging from 0.001-10000 μM for 48 hrs. Results are presented here as Mean±standard deviation of three parallel outcomes of each study

microscopic images of these cell lines showed the stressed morphology, indicating the significant cell death observed at 24 hrs after Genistein treatment (Fig. 3).

Genistein effect was obvious in western blot and flow cytometry thus to give further strength to the above data, caspase 3 activity and expression was checked in

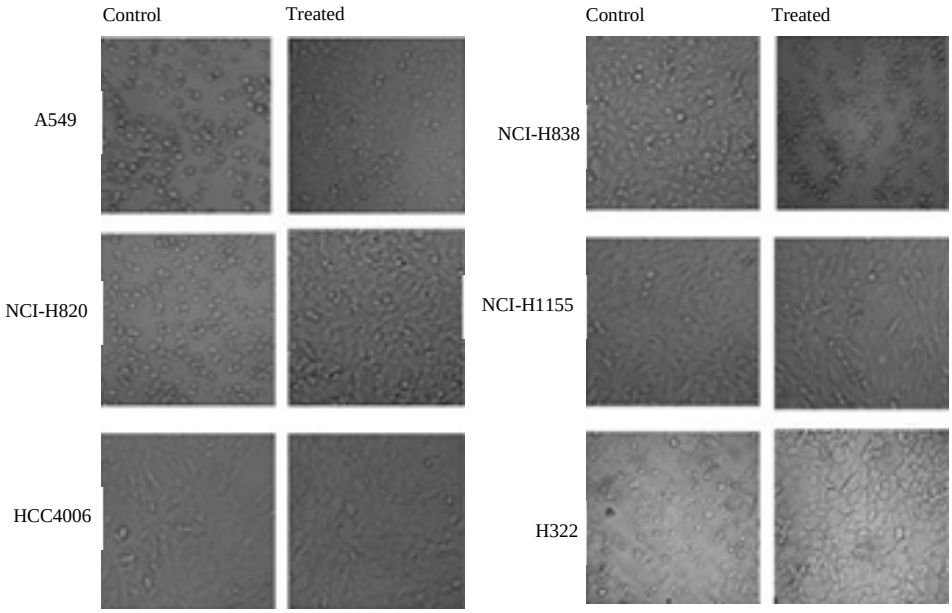

Fig. 3: Morphological changes in Cell line after treatment with genistein (0.3 mM) observed under an inverted microscope (100X)

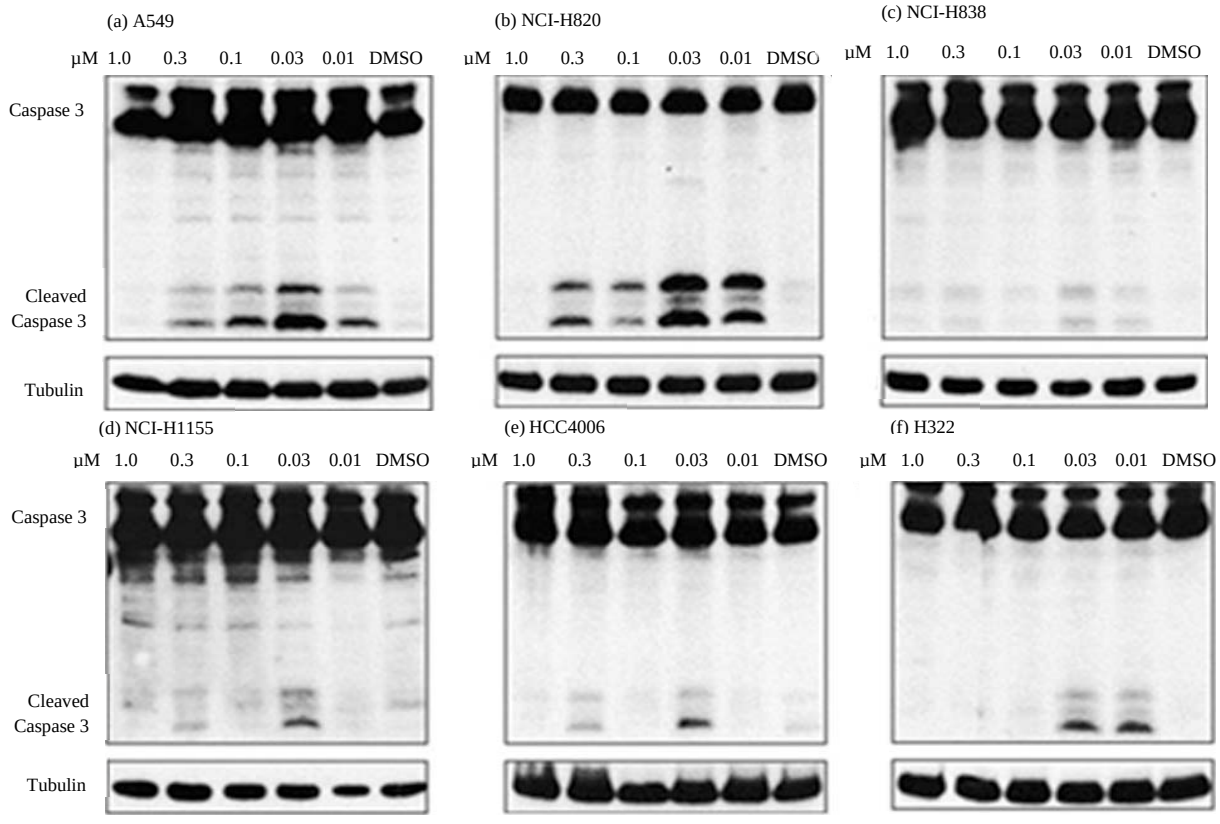

Fig. 4(a-f): Effect of genistein on the expression of modulated and cleaved caspase 3 in different NSCLC cell lines. Western blots of caspase 3 in Non-small cell lung cancer (NSCLC) expressed in (a) A549, (b) NCI-H820, (c) NCI-H838, (d) NCI-H1155, (e) HCC4006 and (f) H322. Tubulin was taken as control/confirmation of blotting

cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 (Fig. 4a-f).

Caspase 3 has been reported as an important mediator in the apoptosis signalling pathway. It has been observed that 48 hrs of Genistein treatment to cell lines under investigation resulted in enhanced caspase-3 and cleaved caspase-3 expression, while the DMSO treatment as control showed no expression for cleaved caspase-3 in all cell lines. In the case of NCI-H820 and A549 was increased and higher than other cell lines signifying that apoptosis was induced by caspase-3 cleavage, while in other cell lines, Genistein might have induced apoptosis through AKT activation or PARP cleavage along with caspase-3 cleavage. Effects of Genistein on EGFR and E-cadherin protein levels in NSCLC cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 were evaluated by treating cells with Genistein for 48 hrs (Fig. 5).

Immunoblots were prepared using antibodies having an affinity for E-cadherin and EGFR as well as antibodies specific to β-actin for loading control. Genistein treatment resulted in a low expression of EGFR in all NSCLC cell lines (Fig. 5). HCC4006 and H322 express light bands for EGFR signifying the low expression of EGFR. This result suggested the Genistein-mediated down-regulation of EGFR in NSCLC cell

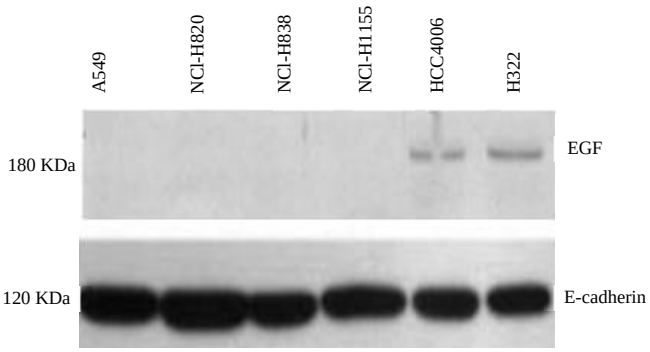

Fig. 5: Effect of genistein on EGFR and E-cadherin protein levels in NSCLC cell lines grown in tissue culture

Antibodies specific to E-cadherin and EGFR was used for Immunoblotting after isolation of proteins from the NSCLC cell line. CCD video camera (Fluorimager 8000, alpha innotech) was used to capture enhanced chemiluminescence (ECL), images

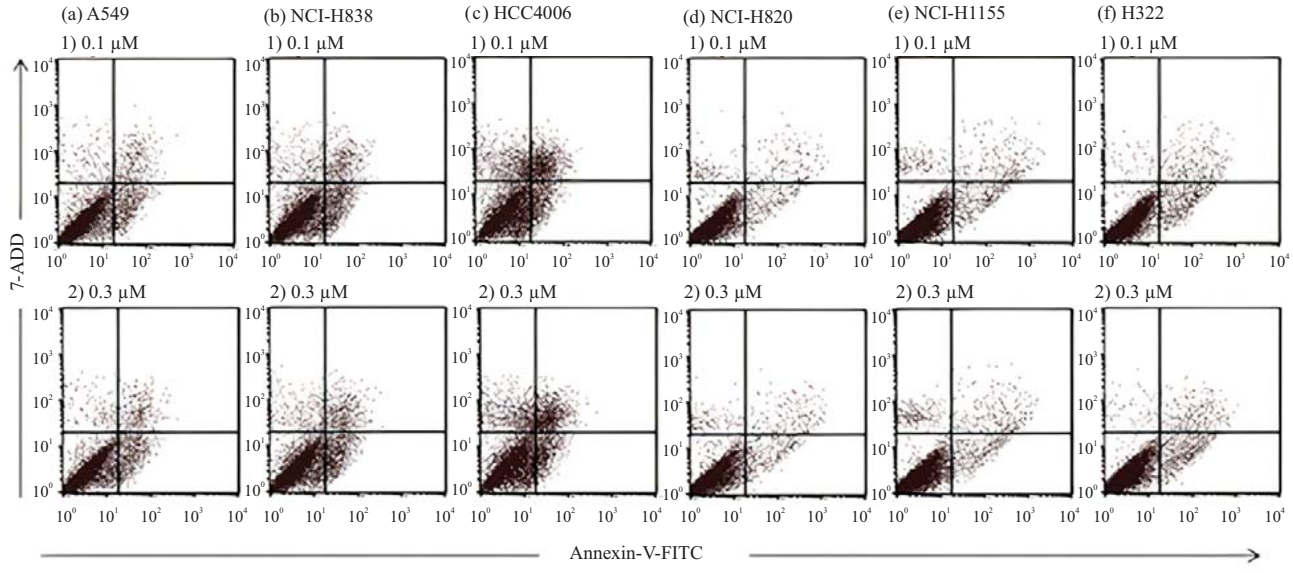

Fig. 6(a-f): Effect of genistein (annexin-V/7-AAD) on induced apoptosis in various NSCLC cell lines viz. (a) A549, (b) NCI-H838, (c) HCC4006, (d) NCI-H820, (e) NCI-H1155 and (f) H322 estimated using flow cytometry.

NSCLC cells were given with 0.1 and 0.3 μM concentrations of genistein and incubated for 24 hrs before the addition of annexin-V-FITC and 7-AAD

lines. Interestingly, treatment with Genistein high levels of E-cadherin over normal expression in all NSCLC, signifying the stimulating effects. As shown in Fig. 5, the NCI-H820 cell line expresses moderately higher levels of E-cadherin than the other NSCLC cell lines. While the H322 cell line expressed low E-cadherin in comparison to other NSCLC cell lines. It might be due to the reason that H322 showed the moderate expression of EGFR after treatment which ultimately results in moderate expression of E-cadherin in them. To further evaluate the effect of Genistein induced apoptosis, NSCLC cell lines A549, NCI-H820, NCI-H838, NCI-H1155, HCC4006 and H322 were analyzed by flow cytometry (Fig. 6a-f).

Results showed that Genistein significantly improved early and late apoptosis when added at 0.1 and 0.3 μM concentrations. Furthermore, these results also suggest that Genistein caused down-regulation of EGFR with high E-cadherin expression. Maximum apoptosis was obtained in case A549 cell line (56.07±3.1%) with early apoptosis at 38.62±2.1% and late apoptosis at 17.45±0.9% cell population treated with 0.3 μM Genistein. These results show that the extent of apoptosis of cells depends on the concentration of Genistein.

DISCUSSION

Genistein, an isoflavonoid, has its abundance in traditional soy products and is largely consumed by the Asian population. The data revealed low incidences of breast and prostate cancer in the Asian population. This isoflavonoid has been reported to modulate cell cycle and apoptosis, reduced dimethyl-benzanthracene-induced mammary adenocarcinoma and increased cell differentiation[16]. At some higher or lower concentration it has its anticancer effect on ovarian, colon, prostate cancer types as well[17,18]. A possible reason for cell death after Genistein treatment observed in the present study is increased expression of caspase-3 in the cell lines. Caspase-3 has been reported as a protease enzyme that catalyzes the trimming of cellular proteins to make them ready for programmed cell death (apoptosis). One study showed that caspase 3 degraded or trimmed DNA-PKcs, PARP and the nucleosome breaking nuclease[19]. In a study, hydroxyl propyl cyclodextrin with Genistein as a combination was treated to a human prostate cancer cell line. A significant decrease in viability of cells at a concentration of 20 and 50 μM of Genistein was observed while in combination with 5 mM hydroxyl propyl cyclodextrin showed apoptotic effect at 2.5 μM and IC50 was 5-10 μM. This value is approximate at the levels of the Genistein plasma concentration of the population taking soy diet as routine[20]. Genistein is a very hydrophilic small size compound while many glycosides are more effective as chemotherapeutic agents when more hydrophobic. So Genistein synthetic derivatives were prepared and checked to its improvement as an anticancer profile. IC50 and LD50 were found to be many folds higher for two derivatives in many cell lines in comparison to core Genistein[21]. Genistein itself has been reported to increase the caspase 12 activity along with calcium increase and μ-calpain (Ca2+dependent proapoptotic proteases) in the breast cancer cell line (MCF-7). In this study, caspase-12 enzyme activity in Genistein treated (apoptotic) cells was done using a peptide ATAD as substrate and its blot was detected using truncated caspase-12 specific monoclonal antibodies while the same was absent in non-apoptotic cells[22]. Genistein at 180 μM concentration reduced the cell vitality effectively i.e., 14.5±3.2 and 13.4±3.1% for HeLa and CaSki cell lines respectively. But Genistein (at ineffective concentration i.e., 80 μM) in combination with cisplatin showed synergistic action and increased the caspase 3 expressions by 231 and 115 % in CaSki and HeLa cells, respectively in comparison to cisplatin alone (6 μM) treatment[23]. Hyperforin and hypericin extracted from St John's wort (*Hypericum perforatum* L.) were also investigated with leukaemia K562 and U937 cells, brain glioblastoma cells LN229 for its antitumor activity. The extract was also checked for the involvement of caspase as the mechanism of action. It was observed that there was a synergistic effect of both the components with and without exposure to light. Hyperforin at 20 μM concentration showed 14.1 fold increase in caspase 9 expression in U937 cells and different levels of expression for other types of caspase enzyme[24]. In earlier studies also, the expression of EGFR resulted in reduced overtly expressed E-cadherin and b-catenin in many cancerous cell line[25]. EGFR and HB-EGF reduced expression of E-cadherin (34 and 26 % respectively), taking H322 cells as control reference[7]. In another study, anti-EGFR monoclonal antibody decreased the movement of H322 and A549 cells due to increased expression of E-cadherin after treatment with mAb. These results also suggested that E-cadherin could act as a biomarker for benign NSCLC cancers[26,27]. Overall, the study implies that E-cadherin could be a biomarker for diagnosis of Non-small Cell Lung Cancer (NSCLC) cancer and our results demonstrate the possible use of Genistein as a chemotherapeutic agent for the treatment of cancer. These findings could be applied in detecting lung cancer and prevent malignancy. Moreover, the discovery could help in the early diagnosis of lung cancer and prevent various malignant tumours. Finally, the study recommends E-cadherin as a potent biomarker for the diagnosis of Non-small Cell Lung Cancer (NSCLC) cancer and Genistein remediated

up-regulation of E-cadherin could prove to be a novel outcome for the treatment of cancer and prevent malignancy. However, the study has certain limitations because there is a need for *in vivo* studies that can analyze the specificity of the therapeutic activities such as toxicity level and mutagenic side effects. Moreover, the results of the present study need to be validated in a large prospective clinical trial. However, the present study provides an insight into preclinical evidence.

CONCLUSION

In this investigation, Genistein could reduce cell viability on various lung cancer cell lines by less than 20%. 0.1 μM concentration of Genistein was enough for 20% cell viability on NCI-H820, NCI-H838 and HCC4006 cell lines. Western blot analysis also revealed the expression of E-cadherin with 0.1 μMGenistein which signifies as a potent biomarker for NSCLC treatment which is confirmed by caspase-3 activity and flow cytometry. Therefore, the study proposes E-cadherin as a potent biomarker for the diagnosis of NSCLC cancer. Moreover, Genistein-mediated up-regulation of E-cadherin could establish as a novel outcome for the treatment of cancer and prevent malignancy.

SIGNIFICANCE STATEMENT

The results in the study showed that Genistein was able to reduce cell viability on various lung cancer cell lines by less than 20%. The western blotting techniques also unveil the expression of E-cadherin with Genistein revealing it as a potent biomarker for the diagnosis of Non-small cell lung cancer. This discovery could help in the early diagnosis of lung cancer and prevent malignancy

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