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Sulforaphane Inhibits Metastatic Events in Breast Cancer Cells through Genetic and Epigenetic Regulation

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Abstract

Metastasis is a deadly event in carcinogenesis, which is regulated both genetically as well as epigenetically. Regulation of key molecules involved in this phenomenon could be a promising strategy in cancer control.

The epigenetic enzyme HDAC6 along with telomerase and HSP90, two genetic markers of carcinogenesis are implicated in the metastatic pathway. Therefore, modulation of these markers may aid in prevention of spread of cancer to distant parts. Plant derived molecules are apparently nontoxic and stud with anticancer activities. Present study aims to investigate the effect of sulforaphane (Sfn), an organosulfur compound on these markers, which might be helpful in inhibition of metastasis.

It was observed that sulforaphane significantly inhibited HDAC6 expression, both at protein and genetic level in metastatic breast cancer cell MDA-MB-231. Inhibition of HDAC6 was associated with increased acetylation of HSP90 and diminished expression of transcription factor c-myc. These results were further confirmed by using tubacin, a specific HDAC6 inhibitor. Activity and expression of human telomerase reverse transcriptase, a main determinant of catalytic activity of the enzyme was found to be inhibited by Sfn. Repression of c-myc led to transcriptional down-regulation of hTERT mRNA and de-repression of p21. Modulation of these proteins led to down-regulation of VEGF and MMPs (2 and 9), two key players of the metastatic event. Regulation of these proteins by Sfn decelerates migration and invasion of the metastatic breast cancer cells, thereby showing anti-metastatic potential.

Sulforaphane, by virtue of its modulatory role on HDAC6 and other associated proteins may lead to inhibition of metastasis in breast cancer cells.

Keywords: HDAC6; HSP90; Telomerase; c-myc; MMPs; MDA-MB-231; Sulforaphane; Metastasis

Introduction

Histone deacetylase 6 or HDAC6, a cytoplasmic, microtubule-associated member of the class IIB HDAC family has unique substrate specificity for nonhistone proteins and is important for both cytoplasmic and nuclear function [1]. This enzyme has been found to be implicated in protein acetylation, regulation of microtubules, growth factor-induced chemotaxis and the processing of misfolded protein aggregates [2]. Two of the major substrates of HDAC6 include α-tubulin and heat shock protein HSP90 [2]. Heat shock proteins (HSPs) are members of the molecular chaperones having essential role in the folding of a large number of cellular proteins. Among several HSPs, HSP90 is an abundant and essential molecular chaperone that regulates the stability and maturation of a wide range of oncogenic client proteins [3,4]. Deacetylation by HDAC6 activates HSP90 to bind with its client proteins. Conversely, inactivation of HDAC6 leading to HSP90 hyper-acetylation, results in loss of chaperone activity of the protein [5]. HDAC6 thereby potentially affects the activity of other client proteins by regulating HSP90 [6]. Several studies have indicated involvement of HDAC6 with prognostic significance in breast cancer [7], association with the invasive potential [8] and better survival of the disease [9]. Elevated expression of HSP90 has been documented in breast ductal carcinomas [10] because of its interaction with several other proteins involved in breast neoplasia. Expression and activity of telomerase, a RNA dependent DNA polymerase is high in 90% of human cancers [11] including breast cancer [12]. Human telomerase reverse transcriptase or hTERT is the catalytic subunit of human telomerase. This protein is known to be over-expressed in human malignancies including breast cancer and is strongly associated with activity of telomerase [13].

Epigenetic modulation of the hTERT core promoter region regulates its transcription [14]. Besides, a number of transcription factors including c-myc have been identified to regulate hTERT promoter directly or indirectly [15]. HDAC6 on the other hand, has been shown to deacetylate β-catenin which leads to activation of c-myc [16]. Inhibition of telomerase and HSP90 therefore becomes an attractive target in cancer research. Inhibitors of HDACs are reported to de-repress human hTERT gene although the underlying mechanisms are yet to be fully explored.

Metastasis or the spread of cancer is a catastrophe in carcinogenesis. Being a late event it often leads to death. Like all other cancers, metastasis is an acute problem in breast carcinoma. There are certain markers that allow the cancer cells to invade and migrate to different parts, culminating in metastasis and hence poor outcome. Inhibition

of this whole event is therefore of enormous importance for a better prognosis.

A group of compounds, known as 'phytochemicals' add flavor and colour to the gourmet; besides they show many medicinal properties including anticancer activities. Epidemiological surveys indicated an inverse correlation between intake of cruciferous vegetables with incidences of cancer [17]. Sulforaphane, a major compound from broccoli/broccoli sprouts possess chemo preventive activity [18]. Sulforaphane has been reported to modulate multiple targets that regulate oxidative stress, induction of apoptosis, cell cycle arrest, inhibition of angiogenesis and metastasis and detoxification of carcinogens [19,20]. This phytochemical has been shown to inhibit breast cancer stem cells by down-regulating the Wnt/β-catenin self-renewal pathway [20]. Previous studies from our laboratory revealed that sulforaphane can induce apoptosis in breast cancer cells via modulation of HSPs [21]. Sulforaphane has been shown to destabilize androgen receptor in prostate cancer cells by inactivating HDAC6 [22].

The present study evaluates the effect of sulforaphane on HDAC6 in metastatic breast cancer cell line MDA-MB-231. A non-tumorigenic breast epithelial cell line (MCF-12F) was used as control. The aim is to observe whether modulation of HDAC6 could decrease the expression and functional activity of HSP90, c-myc and telomerase, leading to inhibition of metastasis.

Materials and Methods

Maintenance of cells and treatment protocol

Human metastatic breast cancer cell line MDA-MB-231 was routinely maintained in DMEM. Normal breast epithelial cell line MCF-12F was maintained in a 1:1 mixture of DMEM and HAM's F_{12} medium with 0.4 mM Ca^{++}, 20 ng/ml epidermal growth factor. Cells were maintained at 37°C in the presence of 15% fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2/95% air. Exponentially growing cells were treated with different concentrations of sulforaphane (1, 5, 10 and 20 μM) and tubacin (2.5, 5 μM) for 24 h.

Western blot analysis

Following sulforaphane treatment, cells were harvested and lysed as described [23]. The lysates were then centrifuged and protein concentration was quantified following Lowry's method. Equally loaded proteins from cell lysates were subjected to electrophoresis on 10% SDS-polyacrylamide gel using electrophoresis buffer and separated proteins were electro-transferred to nitrocellulose membranes using transfer buffer which was followed by proper washing and blocking with 5% BSA. Expressions of HDAC6, HSP90, c-myc, hTERT, p21, VEGF were determined after incubating membranes with specific antibodies. Membranes were thereafter washed with TBST and incubated with alkaline phosphatase conjugated anti-mouse IgG (1:1000 dilutions in TBS). Expressions of proteins were finally detected after addition of BCIP/NBT substrate. β-actin was used as a loading control.

RNA extraction and semiquantitative RT-PCR

Total RNA from the treated cells was isolated by using RNAqueous 4PCR kit (Ambion/Applied Biosystem) following the manufacturer's instructions. Complementary DNA was synthesized from 2 μg of total RNA using RetroScript kit (Ambion/Applied Biosystems). The complementary DNA was then amplified by PCR for 30 cycles with an initial hot start followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 55°C for 30 s and final extension at 72°C for 90 s using forward and reverse primer sequences. For the positive control, a constitutively expressed 'housekeeping' gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken. Amplification products obtained by PCR were separated electrophoretically using 2% agarose gel and visualized by ethidium bromide staining under the Gel Documentation System.

Immunoprecipitation and detection of acetylated HSP90

Immunoprecipitation of HSP90 was performed following the manufacturer's instructions provided in the kit. Briefly, isolated proteins (250 μg each) from cell lysates of the treated and untreated cells were used for immunoprecipitation. Lysate was immunoprecipitated with anti-HSP 90 antibody using sepharose A/G provided. During the incubation period, gentle agitation of the lysate was carried out to allow the target antigen (HSP90) to bind to the antibody. The immobilized immune complexes were eluted from the sepharose A/G column and was immunoblotted using an anti-acetyl lysine antibody.

Chromatin immunoprecipitation assay

Confluent cells were treated with or without 20 μM sulforaphane for 24 h, fixed with 1% formaldehyde at room temperature followed by neutralization after addition of 0.125 M glycine. Cells after proper fixation were harvested and washed at room temperature, followed by lysis and incubation for 30 min in ice. Each sample was sonicated for 5-30 s at an interval of 5 min on ice, followed by centrifugation for 10 min at 4°C. Supernatants were collected and 50 μl of the supernatant was used as an input control during PCR. Sonicated supernatant was further diluted 10 fold in ChIP dilution buffer. Protein-chromatin complexes were immunoprecipitated overnight at 4°C with rotation using mouse control IgG or mouse monoclonal anti-c-myc or mouse monoclonal anti-p21 and were collected on Immuno Pure plus immobilized protein A/G sepharose beads. Beads containing protein-DNA complex was washed for 5 min on a rotating platform using low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl buffer, ice cold TE buffer sequentially. Finally protein-DNA complex was extracted by elution buffer. Samples were extracted twice with phenol/chloroform, and precipitated overnight with ethanol. Centrifugation was done to recover DNA fragments; the pellet was resuspended in double distilled water and used for PCR amplification of the p21 and hTERT gene promoter.

Wound healing assay

Breast cancer cells were grown to confluence in tissue culture plates. A uniform scratch was made in 80% confluent monolayer culture of MDA-MB-231 with a sterile pipette tip. Cells were treated with sulforaphane (20 μM) and incubated for 24 hrs. Migration of cells into the wound was examined by phase contrast microscopy. Photographic images were obtained immediately after scratching and after 24 h in the same location.

Transwell migration and invasion assay

The migration and invasion of MDA-MB-231 cells were determined using a transwell chamber with polyethylene terephthalate

(PET) filter membrane containing insert of 8-µm pore size (Greiner bio one). Serum free cell suspension (2×10^5/well) was placed into the upper chamber. For migration assay the membrane was un-coated, whereas for invasion assay the same was coated with extracellular matrix gel. Lower compartment was filled with medium with 20% FBS as a chemo-attractant. Remaining cells which were neither invaded nor migrated were removed. Migrated/ invaded cells on the underside of the membrane was fixed with formaldehyde (4%) and stained with crystal violet (0.1%). Cells were visualized under bright field microscope.

Gelatin zymography of MMP-2 and MMP-9

Breast cancer cells were incubated in serum-free medium in the presence or absence of sulforaphane for 24 h. Conditioned medium was collected, concentrated and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% gelatin. Following electrophoresis the gel was washed twice in renaturing buffer (pH 7.5, 2.5% Triton X-100), followed by incubation in developing buffer (50 mM Tris-HCl pH 7.5, 5 mM CaCl2, and 200 mM NaCl). The gel was finally stained with 0.5% Coomassie blue R-250 in 30% methanol, 10% acetic acid and finally destained with 30% methanol, 10% acetic acid. Gelatinolytic activities of MMP-2 and MMP-9 were assessed.

Results

Sulforaphane down-regulates HDAC6 in MDA-MB-231 cells

Constitutive expression of HDAC6 was observed in metastatic breast cancer cell line MDA-MB-231 and the results were compared with their normal counterpart MCF-12F. It was observed that expression of HDAC6 is very high in MDA-MB-231 compared to MCF-12F (Figure 1a). Expression of HDAC6 was observed by western blot analysis after treatment of the cells with sulforaphane of different concentrations (1,5,10 and 20 µM) for 24 h. It was observed (Figure 1b) that sulforaphane treatment inhibited HDAC6 expression in a dose dependent manner, indicating an inhibition of this epigenetic marker at the protein level. Furthermore role of sulforaphane on regulation of HDAC6 at transcription level was examined. RT-PCR analysis was performed using specific primers of HDAC6 and the results obtained were represented in Figure 1(c). The results revealed reduced expressions of HDAC6 mRNA by sulforaphane. However, the compound hardly showed any effect on HDAC6 expressions in MCF-12F cells (data not shown). This result supported the fact that sulforaphane inhibited HDAC6 in metastatic breast cancer cells both at protein and at transcriptional level.

Sulforaphane enhances HSP90 acetylation & diminishes c-myc expression

HDAC6 functions as a HSP90 deacetylase. It was therefore important to investigate how down-regulation of HDAC6 by sulforaphane leaves an impact on acetylated HSP90 (Ac-HSP90). Immunoprecipitation of HSP90, followed by immunoblotting with an antibody that recognizes anti-acetylated lysine residue was performed. Result obtained from immunoprecipitation assay revealed enrichment of acetylated lysines on HSP90 with increasing concentrations of the compound (Figure 2a). Expression of total HSP90 was decreased with concentration of sulforaphane. In another set of experiment, cells were treated with tubacin, a specific inhibitor of HDAC6 deacetylase activity. Results showed elevated expression of Ac-HSP90 after treatment with 2.5 and 5 µM tubacin (Figure 2b). These results indicated that up-regulation of Ac-HSP90 may be due to corresponding down-regulation of HDAC6 by sulforaphane. The present findings also suggested that increased acetylation of HSP90 by sulforaphane is due to decrease in the expression of HDAC6. Expression of c-myc has been found to be diminished gradually with increasing concentration of sulforaphane as depicted in Figure 2c. To confirm the involvement of HDAC6 in c-myc regulation, western blot analysis was performed with tubacin (2.5 µM and 5 µM) treated cells for 24 h (Figure 2d). Decrease in c-myc expression was observed with increasing concentration of tubacin, supporting the notion that HDAC6 is a key mediator of c-myc activation. Sulforaphane efficiently inhibited both the protein expression.

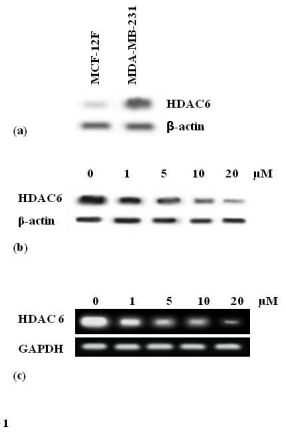

Figure 1: Sulforaphane down-regulates HDAC6 in breast cancer cells (a) Constitutive expression of HDAC6 in normal breast epithelial cells MCF-12F and metastatic breast cancer cells MDA-MB-231 as obtained from western blot analysis revealed a higher expression of protein in cancer cells. β-actin was used as loading control. (b) Cells were exposed to various concentrations of sulforaphane (0, 1, 5, 10 and 20 µM) for 24 h. HDAC6 was analyzed by western blot analysis using specific antibody and was found to be down-regulated by sulforaphane in a dose dependent manner. β-actin was used as control to ensure equal loading of protein. (c) Cells were incubated with sulforaphane (0, 1, 5, 10, and 20 µM) for 24 h to assess mRNA levels of HDAC6. Total RNA was isolated and reverse transcribed. The resulting cDNAs were subjected to PCR with primers and the reaction products were subjected to electrophoresis using 2% agarose gel and visualized by EtBr staining. A decreasing trend at mRNA level was observed after treatment with increasing concentrations of sulforaphane. GAPDH was used as an internal control.

Sulforaphane inhibits hTERT expression

Transcription of hTERT is regulated by c-myc, therefore it is worthwhile to elucidate the role of sulforaphane on hTERT protein and gene transcription. Western blot and RT-PCR techniques using specific primers were conducted as described in Table 1. The result as represented in Figure 3(a) revealed concentration dependent hTERT inhibition in MDA-MB-231 cells. Figure 3(b) indicated significant inhibition of hTERT mRNA by sulforaphane treatment. ChIP assay

was carried out using specific anti-c-myc antibody to ascertain the binding potential of transcription factors c-myc to the endogenous hTERT gene promoter. Negative control for antibodies included diluent and mouse IgG. DNA from cell lysates prior to immunoprecipitation was considered as input. The result as revealed in Figure 3(c) showed that in untreated cells antibodies against c-myc were able to strongly precipitate hTERT promoter indicating the binding of these proteins. On the contrary, no amplified product was detected with control IgG, verifying specificity of antibodies used in this study. Interestingly very poor signal of c-myc were detected after treatment with sulforaphane, suggesting an interaction of c-myc with the hTERT promoter, thereby positively regulating its transcription. The result thus showed that sulforaphane inhibited hTERT gene by preventing the binding affinity of transcription factors c-myc to its promoter.

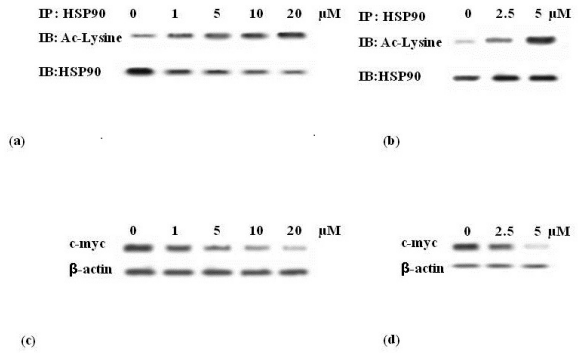

Figure 2: Sulforaphane enhances expression of acetylated HSP90 and decreases c-myc (a) Level of acetylated HSP90 (Ac-HSP90) was studied in MDA-MB-231 cells treated with sulforaphane (0, 1, 5, 10 and 20 μM) for 24 h. HSP90 was immunoprecipitated (IP) from the tumor cell lysates and immunoblotted (IB) with either anti-HSP90 or anti-acetylated lysine (Ac-lysine) antibody. An increase in expression of acetylated lysines on HSP90 was observed. (b) Level of Ac-HSP90 in MDA-MB-231 cells treated with tubacin (0, 2.5 and 5 μM) for 24 h was studied. HSP90 was immunoprecipitated from the cell lysates and immunoblotted with either anti-HSP90 or anti-Ac-lysine antibody and found to be enhanced. (c) The cells were treated with various concentrations of sulforaphane (0, 1, 5, 10 and 20 μM) for 24 h. 50 μg of isolated protein was electrophoresed on 10% SDS-PAGE gel, blotted and probed with antibody against c-myc. A decrease in c-myc expression was observed. β-actin was used as loading control. (d) Differential expression of c-myc protein after treatment with HDAC6 inhibitor tubacin (0, 2.5 and 5 μM) for 24 h also revealed decrease in c-myc expression. For equal loading of protein, β-actin was used as control.

Name	Sequence of Primers
HDAC6	Sense 5'- TGG-CTA-TTG-CAT-GTT-CAA-CCA -3'
	Antisense 5'- GTC-GAA-GGT-GAA-CTG-TGT-TCCT -G-3'
hTERT[a]	Sense 5'-AGC-CAG-TCT-CAC-CTT-CAA-CC-3'
	Antisense 5'-GTT-CTT-CCA-AAC-TTG-CTG-ATG-3'
p21	Sense 5′-GCA-GAC-CAG-CAT-GAC-AGA-TTT-3′
	Antisense 5′-GGA-TTA-GGG-CTT-CCT-CTT-GGA-3′
GAPDH[b]	Sense 5'-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3'
	Antisense 5'-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3'

[a]hTERT: Human telomerase reverse transcriptase

[b]GAPDH: Glyceraldehydes 6 phosphate dehydrogenase

Table 1: Oligonucleotide used in RT-PCR.

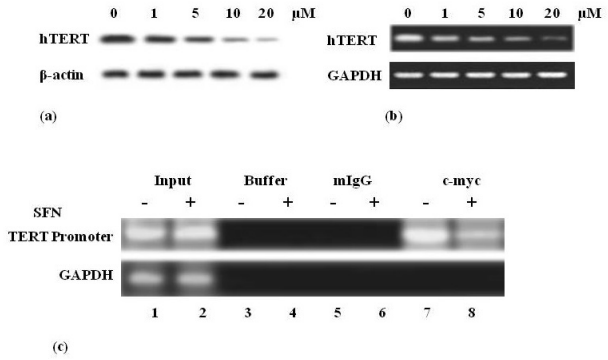

Figure 3: Sulforaphane inhibits hTERT at the protein and mRNA level in breast cancer cells (a) Expression of hTERT protein in breast cancer cells was determined by western blot analysis after treatment with sulforaphane (0, 1, 5, 10 and 20 μM) for 24 h and was found to be diminished. β-actin was used as loading control. (b) Expression of mRNAs was carried out following RT-PCR technique. RT-PCR was performed using specific primers as mentioned in Table 1. The amplified PCR products were run in a 2% agarose gel and visualized by EtBr staining. mRNAs had been found to be down-regulated after treatment of cells with different concentrations of sulforaphane as mentioned before. GAPDH was used as a house-keeping control gene. (c) ChIP (Chromatin immunoprecipitation) was employed to detect c-myc and hTERT gene promoter complex in response to sulforaphane. Cells were treated with or without sulforaphane (20 μM) for 24 h followed by ChIP with control or specific antibodies. Sulforaphane inhibited hTERT gene by preventing the binding affinity of transcription factors c-myc to its promoter. The DNA hTERT promoter and human GAPDH gene were detected by PCR using specific primers.

Sulforaphane represses cyclin dependent kinase inhibitor protein p21

Repression of p21 is influenced by c-myc. Western blot analysis revealed increase in the expression of p21 with increasing concentration of sulforaphane (Figure 4a). ChIP assay was performed to assess whether sulforaphane could induce the release of c-myc repression from p21 gene promoter. Breast cancer cells were immunoprecipitated with specific antibody against c-myc and were subjected to PCR amplification using primers specific for p21 promoter fragment (Table 1). c-myc was able to precipitate p21 promoter in untreated cells, indicating binding of c-myc with the promoter (Figure 4b). Very poor binding ability of c-myc on the p21 promoter region was observed in sulforaphane treated cells. On the contrary no amplified product was detected with control IgG which

verifies the specificity of c-myc binding. This result further strengthened the fact that c-myc plays a role in repression of p21 expression and down-regulation of this transcription factor by sulforaphane led to de-repression of p21 in metastatic breast cancer cells.

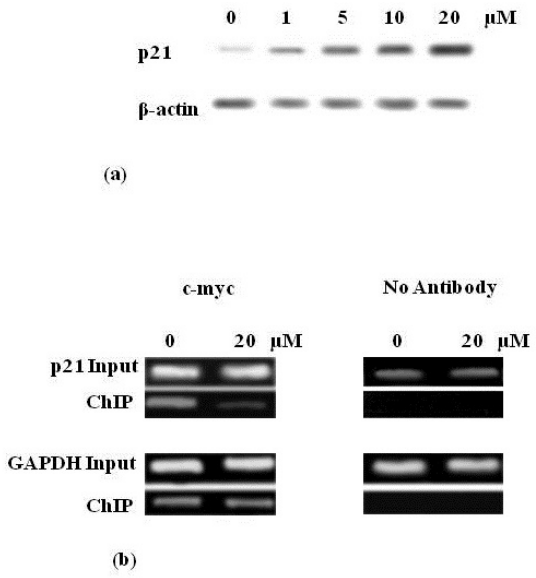

Figure 4: Cell cycle inhibitory protein p21 gets repressed by sulforaphane in breast cancer cells. (a) Exponentially growing cells were exposed to different concentrations of sulforaphane for 24 h. Cells were harvested, total proteins were extracted and 50 µg protein was subjected to western blotting using antibodies against p21. Results showed repression of p21 with increasing concentrations of sulforaphane. β-actin was used as control to ensure equal loading of protein. (b) Gene promoter complex of c-myc and p21 were detected in response to sulforaphane. c-myc antibody was used to immunoprecipitate soluble chromatins from breast cancer cells cultured either with or without sulforaphane (20 µM) for 24 h. DNAs were immunoprecipitated and subjected to PCR using primers for the p21. Very poor binding ability of c-myc to the p21 promoter was observed. GAPDH gene was used as housekeeping gene. The results obtained are representatives of two or more experiments from independent immunoprecipitations.

Sulforaphane reduces motility, migration and invasion of metastatic breast cancer cells

To analyze the effect of sulforaphane on cell migration, wound healing (scratch) assay was performed. Cell motility following wound generation is an indicator of cell migration. Presence of sulforaphane was found to reduce cell migration. Figure 5a revealed that after 24 h, appreciable closure of wound was observed in untreated cells, but not in cells treated with 20 µM sulforaphane. Width of the wound was measured and percentage of wound closure was denoted in the bar diagram. The ability of sulforaphane to reduce the migration (Figure 5b) and invasiveness (Figure 5c) of MDA-MB-231 cells was further ascertained by the Boyden chamber assay. Quantitative measures of migration and degree of invasiveness had been shown in the adjacent bar diagrams respectively. The results revealed that migratory and invasive potential of MDA-MB-231 cells were negatively affected. Present findings therefore indicated that HDAC6 inhibition facilitates prevention of cell motility, migration and invasion.

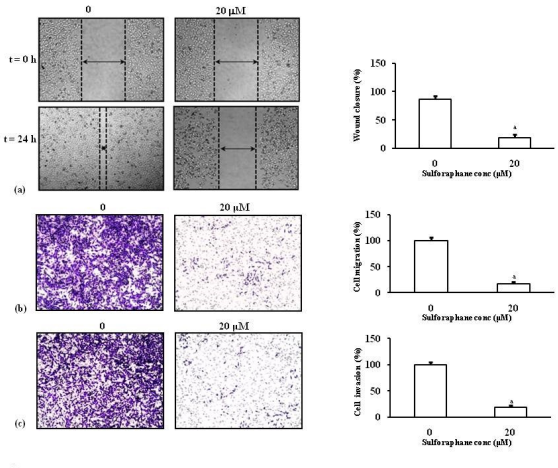

Figure 5: Sulforaphane inhibits metastatic properties of breast cancer cells. (a) Wound healing assay was initiated by a uniform scratch in a pertidish containing MDA-MB-231 cells grown in confluence. Scratch was monitored under a microscope. The width of the scratch was measured and the percentage of closure was estimated. Results, expressed as mean ± SE (n=3) shows a significant inhibition of wound closure [a]($p<0.001$) by sulforaphane. (b) For migration assay, MDA-MB-231 cells were seeded into the upper chamber of the transwell system. Cells were treated with suforaphane (20 µM) for 24 h and allowed to migrate. Migrated cells were fixed, stained with crystal violet and photographed. Acetic acid was used to extract cells bound to crystal violet. Optical absorbance was measured to quantitate the extent of cell migration, which was represented as percentage of migrated cells. Results are expressed as mean ± SE of three independent experiments. It was indicated that sulforaphane had an inhibitory effect on tumor cell migration. Extent of migration is distinctly different from the untreated cells, with significance level [a]($p<0.001$). (c) Effect of sulforaphane (0, 20 µM) on the invasiveness of the metastatic breast cancer cells was studied using invasion assay. Treated cells were seeded in a transwell unit containing a membrane coated with ECM gel. Cells that have invaded through the ECM gel were fixed, stained with crystal violet and photographed. Quantification of invasiveness, as revealed by the absorbance of eluted crystal violet indicated reduction of invasive property of breast cancer cells. Bar diagrams represent percent of invaded cells. Results are expressed as means ± SE (n=3) and are significant compared to the untreated cells [a]($p<0.001$).

Sulforaphane inhibits metastatic proteins

Effect of sulforaphane on gelatinolytic activity of MMPs (2 and 9) and expression of VEGF was examined. MDA-MB-231 cells were grown in absence and presence of sulforaphane for 24 h. It was observed that sulforaphane inhibited the activity of these MMPs in a dose dependant manner (Figure 6a), maximum effect was observed at 20 µM. Corresponding intensities from zymography are quantitative

indicators of MMP activity and are shown in Figure 6b. Expression of VEGF was also found to be inhibited by sulforaphane (Figure 6c). Band intensities corresponding to western blot bands are depicted in Figure 6d. Results indicated inhibition of metastasis in MDA-MB-231 cells by sulforaphane.

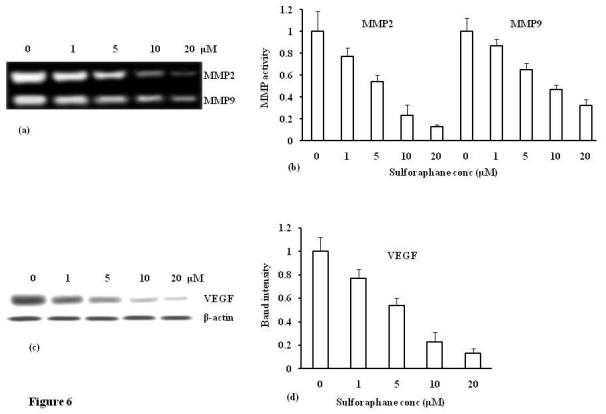

Figure 6

Figure 6: Sulforaphane inhibits metastatic proteins (a) Effects of sulforaphane on the gelatinolytic activities of matrix metalloproteinases were studied by zymography. Cells were treated with various doses of sulforaphane (0, 1, 5, 10, 20 μM) for 24 h and activities of the MMPs were accessed using the conditioned media collected from cells. Results showed a decrease in MMP activities. (b) MMP activities as influenced by sulforaphane are depicted in figure showing a quantitative estimate of inhibition of these proteins. (c) Expression of VEGF as influenced by sulforaphane was studied by western blot analysis. Cells were treated as mentioned previously. Cell lysates were subjected to western blot analysis using VEGF antibody, where decrease in the expression was indicated. β-actin was used as loading control. (d) Quantification of VEGF expression as modulated by sulforaphane has been depicted in bar diagram. Results indicated inhibition of VEGF expression by sulforaphane.

Discussion

Histone deacetylase 6 (HDAC6), an epigenetic regulator and a member of the class IIB HDAC family has gained popularity as a target in cancer prevention and therapy due to its major contribution in oncogenic cell transformation. Abundant expression of HDAC6 imparts cell survival, hence poor prognosis in malignancy [6]. Therefore inhibition of HDAC6 is very important in control of carcinogenesis. This protein has also been found to regulate heat shock protein 90 (HSP90), which is also of great implication in cancer development.

HDAC6 and HSP90 are major contributors of metastasis; their upregulation imparts enhanced cell migration and invasion to distal parts. Therefore, expressions of these two markers are found to be high in metastatic breast cancer cells. Sulforaphane, a natural glucosinolate exerted its anticancer activity by inhibiting HDAC6 both at the protein and genetic level. Similar results had been observed in prostate cancer cells where this natural organosulfur compound attenuated AR signaling by inactivating HDAC6 [22]. HSP90 plays a pivotal role in cell signalling by promoting the activity and stabilisation of signalling proteins [24]. HDAC6 by virtue of its deacetylation action on HSP90 leads to hyperacetylation of the protein, culminating in the loss of the chaperoning function. Present findings clearly show that sulforaphane inhibits HDAC6, concomitantly increasing the acetylation of HSP90. Sulforaphane was earlier reported to interact with specific amino acid residues of HSP90, disrupting HSP90-cochaperone complex and thereby inhibited the chaperoning function of the protein and induced degradation of HSP90 client proteins [25].

Previous studies reported that HDAC6 promotes c-myc expression, resulting in the proliferation of ER-negative breast cancer cells [16,26]. Over expression of HDAC6 was found to be associated with enhanced c-myc expression. Therefore, c-myc and HDAC6 were considered as potential therapeutic targets [27].

The hTERT gene, also known as human telomerase gene is essential for carcinogenesis and is highly expressed in cancer resulting in stabilization of telomere and immortalization of cells [28]. hTERT regulation is a remarkably complex process and proximal region of hTERT promoter contains a number of consensus sequences, including binding sites for c-myc [29]. Since c-myc plays a crucial role in hTERT transcription, the effect of sulforaphane on hTERT protein expression and gene transcription was determined following western blot and RT-PCR technique. Sulforaphane, in this study inhibited hTERT gene expression as a result of repression of binding affinity of c-myc to TERT promoter sequence. p21 is an important cell cycle inhibitory protein and c-myc has been reported to repress p21 expression both at transcriptional [30] and post-transcriptional level [31]. Present findings suggest that c-myc was able to precipitate p21 promoter in untreated cells, indicating binding of c-myc with the promoter (Fig. 4b). Very poor binding ability of c-myc on the p21 promoter region was observed in sulforaphane treated cells. On the contrary no amplified product was detected with control IgG verifying the specificity of c-myc binding. This result further corroborates the fact that c-myc plays a role in repression of p21 expression. Down-regulation of this transcription factor by sulforaphane led to de-repression of p21 in metastatic breast cancer cells.

Cytoplasmic enzyme HDAC6 plays an important role in the regulation of cell migration and angiogenesis [32,33], which is crucial for cancer metastasis. Therefore inhibition of HDAC6 might prevent the metastatic event. It was observed that migration, invasion and motility of breast cancer cells were hindered by sulforaphane. Therefore to ascertain the anti-metastatic role of sulforaphane, several metastasis related proteins had been studied. Matrix metalloproteinases play pivotal roles in migration and invasion. Gelatin zymography results revealed that activity of MMP2 and MMP9, two important metastatic proteins had been diminished by sulforaphane. VEGF, a proangiogenic factor plays a key role in the development of tumorigenesis [34]. VEGF expression as reflected in western blot results showed a downward trend with increasing concentration of sulforaphane.

In conclusion, sulforaphane, a natural glucosinolate significantly inhibited HDAC6, an important enzyme belonging to class IIb family at transcriptional and translational level. Inhibition of HDAC6 was associated with induction of acetylation of HSP90 and down-regulation of transcription factor c-myc. Attenuation of c-myc expression was linked with specific inhibition of hTERT. It was apparent that sulforaphane efficiently decreased c-myc binding ability

to the hTERT promoter region, thereby repressing genetic transcription and inhibition of telomerase activity. Treatment with sulforaphane ultimately released c-myc from the endogenous p21 promoter region, causing increased expression of p21 in metastatic breast cancer cells, independent of p53 expression. Inhibition of HDAC6 ultimately inhibited metastatic property of breast cancer cells and that too by inhibition of metastatic proteins. The pictorial representation of the metastatic event has been shown in Figure 7. HDAC6 may regulate metastasis directly or indirectly. The control of metastasis may be achieved through regulation of HSP90 by HDAC6. HDAC6 may also regulate c-myc, which finally controls metastasis by regulation of hTERT and p21. Sulforaphane, by virtue of its inhibitory action on HDAC6 and other associated proteins in the trail of progression may aid in metastasis control.

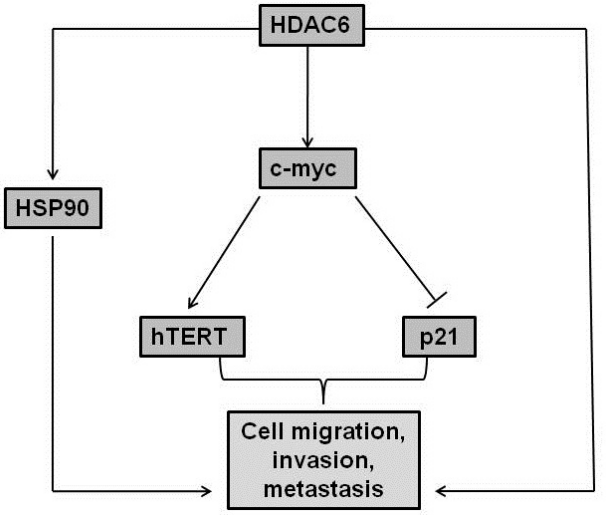

Figure 7: Probable pathway in the development of metastasis has been shown schematically.

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