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Demethylationof Promoter Region of FHIT Gene in A549 Cell Line by Sweet Potato Leaf Extract

Rahul Dev Verma[1], Manish Katiyar[1],Urvashi Verma[1],Gitanjali Sharma[2], Anju Shrivastava[2*], Abhimanyu Kumar Jha[1*]

1- Department of Biotechnology, IMS Engineering College, Ghaziabad, Uttar Pradesh, India.
2- Department of Zoology University of Delhi, Delhi, India.
- Corresponding authors (e.mail:- abhimanyu2006@gmail.com, anjubhu@yahoo.com.)

ABSTRACT

Epigenetics has recently emerged as an advanced and emerging area of molecular biology. Epigenetic silencing of tumor suppressor genes is emerging as a well-established oncogenic process. Since, epigenetic changes can be reversed, the present study was designed to check the demethylation of promoter region of FHIT, a tumor suppressor gene, in A549 cells, after treatment with ethanolic extract of sweet potato leaves. After calculating the IC_{50} value, the cells were treated with 5 mg/ml (IC_{50} value). The present study shows that demethylation of FHIT promoter was observed after 48 h of treatment and this demethylation increases as the time period of treatment was increased to 72 h.

INTRODUCTION

Epigenetic changes such as DNA methylation act to regulate gene expression in normal mammalian development. Promoter hypermethylation also plays a major role in cancer through transcriptional silencing of critical growth regulators such as tumor suppressor genes. Other chromatin modifications, such as histone deacetylation and chromatin-binding proteins, affect local chromatin structure.There are several demethylating agents currently being evaluated in preclinical and clinical studies. 5-aza-cytidine and 5-aza-2-deoxycytidine are the most studied. Some other drugs such as procainamide are also in different stages of trial **(Gonzalez et al., 2005**).Most of the synthetic compounds have cytotoxic effects. Hence, the focus is on natural products for the epigenetic reversal. Phytochemicals derived from fruits and vegetables, are referred to as chemo preventive agents and include curcumin, 6-

gingerol, ursolic acid, silymarin, anethol, catechins,engenoletc.**(Dorai and Aggarwal, 2004)**. These chemopreventive agents have potential to be used in current cancer therapies **(Dorai and Aggarwal, 2004)**. Epigallocatechin-3-gallate (EGCG) has been shown to cause demethylation of the CpG islands in the promoters and reactivation of methylation silenced genes such as *p16INK4a,* retinoic acid receptors in human oesophageal, colon, prostate, and mammary cancer cell lines **(Fang et al., 2003)**.

DNA methylation, primarily at the C_5 position of cytosine, affects gene expression in many biological processes such as differentiation; genomic imprinting, DNA mutation, and DNA repair. DNA hypermethylation, usually occurring at promoter CpG islands, is a major epigenetic mechanism in silencing the expression of genes. The importance of promoter hypermethylation as well as global hypomethylation in carcinogenesis has been extensively discussed.Epigenetic events play a significant role in the development and progression of cancer. Mutations occurring in oncogenes frequently result in a gain of function, while mutations or deletions associated with tumor suppressor genes cause a loss or inactivation of negative regulators. Loss of function, however, can also occur through epigenetic changes such as DNA methylation. 'Epigenetics' refers to heritable changes in gene expression that do not result from' alterations in the gene nucleotide sequence. When DNA is methylated in the promoter region of genes, where transcription is initiated, genes are inactivated and silenced.This process is often disregulated in tumor cells. In cancer, epigenetic silencingthrough methylation occurs at least as frequently as mutations or deletions and leads to aberrant silencing of normal tumor-suppressor function. There are a number of tumor types, particularly hematopoietic malignancies such as myelodysplastic syndromes (MDSs), in which hypermethylation occurs and results in leukemic transformation.The DNA methylation inhibitors azacytidine and decitabine can induce functional re-expression of aberrantly silenced genes in cancer, causing growth arrest and apoptosis in tumor cells.These agents, along with inhibitors of histone deacetylation, have shown clinical activity in the treatment of certain hematologic malignancies where gene hypermethylation occur.Since epigenetic changes are reversible, inhibitors of DNA methylation such as azacitidine (5-azacytidine) and decitabine can derepress silenced tumor suppressor genes and restore their normal function.The therapeutic application of these methylation inhibitors is providing new and effective options for patients with leukemias and related diseases.

Plants with anticancer effect

Plant is the major source of products which are used for medicinal purpose in treatment of various kind of cancers. Green tea extracts and its catechins,especially EGCG are used against chemical carcinogens (**Yang et al.,2009**).Green tea(*Camellia sinensis*) catechins especially EGCG inhibit lung tumour formation,which has been demonstrated in different animal model,including those induced by tobacco smoke related chemical carcinogen such as 4-(3-methylnitrosamino)-1-(3-pyridy)-1-butanone,and N-nitrosodimethylamine(**Ju et al.,2007**). EGCG,also inhibit the prostate cancer and its metastasis (**Ju et al.,2007**).The administration of EGCG at 0.02%-0.32% reverse the hypermethylation of tumour suppressor gene like *p53*(**Yang et. al., 2009**).

Sweet potato leaves are an excellent source of dietary polyphenols such as anthocyanins and phenolic acid.The polyphenols rich sweet potato green extract (SPGE) exerts antiproliferative activity in prostate cancer cell lines (**Karna et al., 2011**).

MATERIALS AND METHODS

Collection of cell line

The Lung cancer cell line,A549,was obtained from the National Centre for Cell Sciences (Pune,India).

Cell Culturing

A549 alveolar lung cancer cell lines were cultured according to standard protocols (**Freshney, 1994**). Briefly, the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS in 5% CO_2 at 37 °C. The cells were re-supplemented with fresh medium and ethanolicplant extracts every 48 h.

Preparation of Sweet Potato Plant Extract

Sweet potato leaves were obtained from the agricultural field. Leaves of the sweet potato were shade dried and powdered.Powdered leaves were taken in petriplate and soaked into the absolute alcohol.Extracts were prepared by soaking powdered leaves in ethanol overnightfor three consecutive days.The supernatant was collected daily and finally concentratedinpetriplates. Last two steps were repeated 2 times to collect the extract.Now the ethanol extract was dried in incubator.Dried powder from ethanol was dissolved in DMSO.

Crystal Violet Test

The cell viability and cytotoxicity of the lung cancer was studied by the Crystal violet test (**Ishiyama et. al., 1996**).

DNA Isolation from Cell line

DNA was isolated by using the Phenol-Chloroform-Isoamyl alcohol method (**Jha et. al., 2010**).

Sodium Bisulphite Modification

The isolated DNA was modified by Sodium bisulphite modification. The EZ DNA methylation kit for the sodium bisulphite modification was obtained from Zymo Research Corporation(USA).

Methylation Specific PCR:(MS- PCR)

After this, MS-PCR of sodium bisulphite modified DNA was carried out using methylation specific primers (Table 1). Methylation-specific PCR is a sensitive method to amplify and detect a methylated region of interest using methylated-specific primers on bisulphite-converted genomic DNA.

Table 1: Primer sequences

S.NO.	PRIMER NAME	SEQUENCE (5'-3')	VOLUME (100pmol/µl)	Tm (deg. celsius)
1	FHIT Methylated F	5'-CGT AAA CGA CGC CGA CCC CAC TA-3' (23)	388	62.6
2	FHIT Methylated R	5'-TTG GGG CGC GGG TTT GGG TTT TTA CGC-3' (27)	343	65.0
3	FHIT Unmethylated F	5'-TTG GGG TGT GGG TTT GGG TTT TTA TG-3' (26)	373	62.0
4	FHIT Unmethylated R	5'-CAT AAA CAA CAC CAA CCC CAC TA-3' (23)	343	58.0

Annealing Temperature

The optimum annealing temperature was found to be 60^{o}C for both methylated and unmethylated DNA.

RESULTS

The cells were observed morphologically.Fig.1ashows the untreated A549 cells. The cells were observed to be spindle shaped. The DNA was isolated from these cells.The genomic DNA was run on 1% agarose gel. The DNA was found to be intact (Fig.2).

Now the treatment was given and results were observed using a proper control. The treatment was given at two concentrations, 5mg/ml (IC_{50} value) and 10 mg/ml, out of which the 10mg/ml concentration resulted in more apoptosis (Fig.1b and 1c).Fig 1b showsmorphology of cells after treatment with 5mg/ml ethanolic extract of sweet potato.Fig 1c showsmorphology of cells after treatment with 10mg/ml ethanolic extract of sweet potato.

DNA was isolated from A549 cells after treating cells with sweet potato extract at 5mg/ml for different time intervals viz 48 h, 72 h and 6 days (Fig.3). After 6 days of treatment, we could not find the DNA band in agarose gel electrophoresis. It may be because most of the cells may have died due to apoptosis.Now the MSP was performed to check the reversal of hypermethylation.

This result showed that the demethylation of FHIT promoter was taking place after treatment with ethanolic extract of sweet potato leaves for 48 h and this demethylation was found to increase after 72 h of treatment (Fig.4).

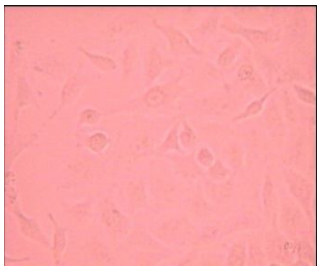

Fig 1a:Morphology of A549 Cells

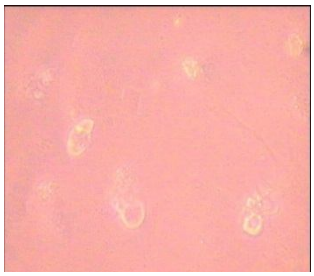

Fig 1b:Treatment with 5mg/ml ethanolic extract.

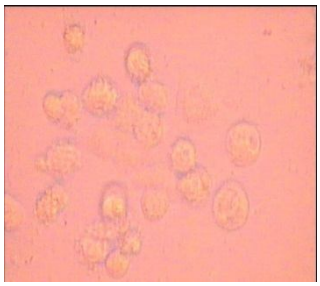

Fig 1c: Treatment with 10mg/mlethanolic extract

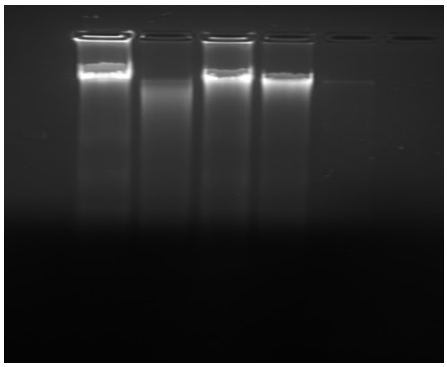

Fig 2:Genomic DNA isolated fromA549 cells

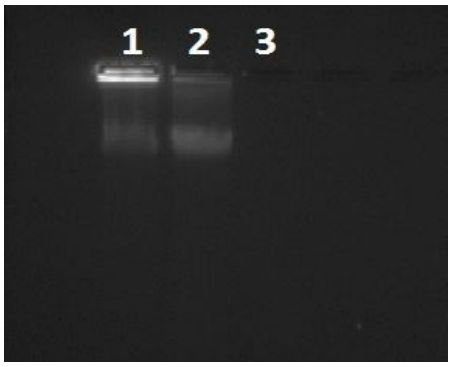

Fig 3: Isolated DNA (1) 48h, (2) 72h (3) 6 daysafter treatment with ethanolic extract

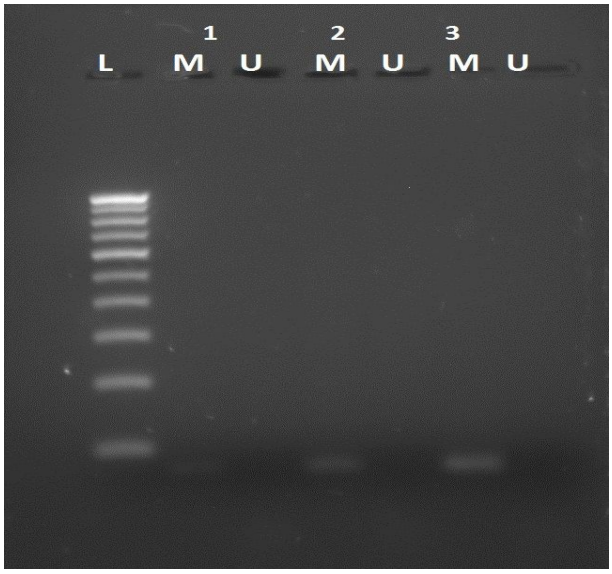

Fig 4: Methylation Specific Band (MSB) after (1) 72 h, (2) 48 h of treatment with the sweet potato extract. The lane (3) represents the control (untreated cells).

DISCUSSION

Many contemporary studies have been carried out on the epigenetic changes resulting in many types of neoplasia and their possible reversal using natural compounds. It has been concluded that epigenetic changes such as DNA methylation can be reversed.

Epigenetic changes like DNA methylation play an important role in several biological processes like cancer, aging & development. Epigenetics has reached a new level of maturity over the past few years, with many findings highlighting the intimate link between DNA methylation and histone modifications. As a result of these discoveries, we have begun to unlock the long-standing mystery of how CpG methylation patterns are established.

According to a recent study, several phytochemicals cause the reversal of hypermethylation and reactivation of tumor suppressor gene. **Jha et al. (2014)** showed the reversal of hypermethylation of *RARβ2* gene in SiHa cell line by *Withaniasomnifera*. It has already been shown that curcumin and genistein cause reversal of hypermethylation and reactivation of *RARβ2* gene (**Jha et al., 2010**).

After calculating the IC_{50} value for the plant extract the treatment was given at 5mg/ml. The apoptosis was observed in the cells. Apoptosis is programmed cell death. At the IC_{50} value of the extract for the cells, 50% of the cells died. The sweet potato extract with which the cancerous cells were treated had the particular component which caused the reversal of the methylated promoter of FHIT gene in cancer cells.

Several dietary polyphenols have been shown to cause the reversal of the methylated DNA in cancer cell lines (**Fang et al., 2003, 2007**).

After treating the A549 cells with sweet potato extract the apoptosis was observed in the cells after 48 h of treatment, and this increased with the increase in time interval of treatment i.e. 72 h. The cells were treated with the sweet potato extract for 48h, 72h and 6 days. The DNA was isolated from the treated and untreated cells after treatment with the plant extract for different intervals. In case of the DNA sample obtained after 6 days treatment, the DNA concentration was found to be very less because of less number of living cells. This was followed by sodium bisulphite modification. After carrying out MSP, it was observed that the reversal of hypermethylation takes place in FHIT, a tumor suppressor gene. This demethylation increased with increase in the duration of treatment from 48 h to 72 h.

CONCLUSION

It can be concluded that the sweet potato has an ability to cause reversal of promoter hypermethylation of FHIT gene and may lead to its reactivation. Much study needs to be carried out to screen some other novel compounds and plant extracts which can cause demethylation of promoter region of tumor suppressor genes.

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