Original Article

Reversal of Hypermethylation and Reactivation of the *RARβ2* Gene by Natural Compounds in Cervical Cancer Cell Lines

(cytotoxicity / dietary polyphenols / demethylation / IC_{50} / MSP)

A. K. JHA[1], M. NIKBAKHT[1], G. PARASHAR[1], A. SHRIVASTAVA[2], N. CAPALASH[1*], J. KAUR[1*]

[1]Department of Biotechnology, Panjab University, Chandigarh, India
[2]Department of Zoology, University of Delhi, Delhi, India
*The authors contributed equally.

Abstract. Reactivation of tumour suppressor genes that have been silenced by promoter methylation is a very attractive molecular target for cancer therapy. The treatment of a squamous cervical cancer cell line, SiHa, with 20 µM curcumin and genistein resulted in demethylation of promoter of the *RARβ2* gene and led to the reactivation of the gene. The degree of methylation as observed by MSP decreased as the time period of treatment was increased from 72 h to 6 days. In HeLa cells (an adenocarcinoma cervical cancer cell line) there was also reversal of hypermethylation of the *RARβ2* gene after six days of treatment with 20 µM curcumin. However, allyl sulphide treatment (20 µM) did not cause the reversal of hypermethylation until 72 h of treatment in the SiHa cell line. This is the first report to show the reversal of hypermethylation of the *RARβ2* gene by genistein and curcumin in cervical cancer cell lines. Furthermore, these compounds acted as double-pronged agents as they caused apoptosis in the treated cervical cancer cell lines in addition to reversal of promoter hypermethylation.

Introduction

Epigenetic silencing of tumour suppressor genes is emerging as a well-established oncogenic process and reactivation of tumour suppressor genes that have been silenced by promoter methylation is a very attractive molecular target for cancer therapy (Gonzalez et al., 2005). 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 and were developed over 30 years ago as classical cytotoxic agents, but were subsequently discovered to be effective DNA methylation inhibitors. Zebularine is a new oral cytidine that has been shown to cause demethylation and reactivation of the silenced and hypermethylated *p16* gene (Angeles et al., 2005). Some other drugs such as procainamide and hydralazine are also in different stages of trial (Gonzalez et al., 2005).

As most of the synthetic compounds may have cytotoxic effects, the focus is on natural products for the epigenetic reversal. Phytochemicals derived from fruits and vegetables, referred to as chemopreventive agents, include genistein, diallyl sulphide, S-allyl cysteine, allicin, lycopene, curcumin, 6-gingerol, ursolic acid, silymarin, anethol, catechins and engenol (Dorai and Aggarwal, 2004). These chemopreventive agents have potential to be used as adjuncts to 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 $p16^{INK4a}$, retinoic acid receptor ß, O^6-methylguanine methyltransferase, human mutL homologue 1, and glutathione *S*-transferase-π in human oesophageal, colon, prostate, and mammary cancer cell lines (Fang et al., 2003). The demethylating activity can be enhanced by the presence of histone deacetylase inhibitors or by longer-term treatment (Fang et al., 2003; 2007).

Soy-derived genistein and lycopene from tomato have been shown to alter gene expression in ways that can either promote or potentially inhibit the carcinogenic processes in breast cancer cell lines. Both genistein and lycopene, at very low, dietarily relevant concentrations can potentially mitigate tumorigenic processes via promoter methylation modulation of gene expression (King-Batoon et al., 2008). Not much data is available on reversal of promoter hypermethylation by natural compounds in cervical cancer cell lines.

Received July 6, 2010. Accepted September 9, 2010.

Financial assistance to AKJ, NC and JK was provided by CSIR – Council of Scientific and Industrial Research, India.

Corresponding author: Jagdeep Kaur, Department of Biotechnology, Panjab University, Sector-14, Chandigarh-160014, India. Phone: (+91) 172-2534086; Fax : (+91) 172-2541409; e-mail: jagsekhon@yahoo.com

Abbreviations: DNMT – DNA methyltransferase, EGCG – epigallocatechin-3-gallate, MSB methylation-specific band, UMSB – unmethylation-specific band.

Material and Methods

Material

The cervical cancer cell lines, SiHa and HeLa, were procured from the National Centre for Cell Sciences (Pune, India). Curcumin and allyl sulphide were purchased from Acros Organics (Fair Lawn, NJ). Genistein and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from HiMedia (Mumbai, India). 5-aza-2-deoxycytidine and trizol reagent were obtained from Sigma Chemicals (Pvt) Ltd. (St. Louis, MO).

Culture of cell lines

SiHa and HeLa 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 test compounds every 48 h.

Cytotoxicity of chemopreventive agents

The cytotoxicity of curcumin, genistein and allyl sulphide was studied on the cervical cancer cell lines by the MTT method (Heckenkamp et al., 1999). MTT assay was carried out to estimate cell viability after treatment with the test compounds. Briefly, the cells were cultured in 96-well plates at a density of 2.5×10^4 cells per well in the presence of the above-mentioned compounds. After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37 °C and 5% CO_2 for 2 h. The water-insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was read by a microplate reader at a wavelength of 570 nm.

Morphological changes

Morphological changes in SiHa cells were observed through a phase-contrast microscope after 24 h of treatment with curcumin and genistein and allyl sulphide at IC_{50} values along with proper controls.

Ethidium bromide staining

SiHa cells were stained with ethidium bromide (100 µg/ml) after treatment with different natural compounds (50 µM) for 96 h and observed under a fluorescence microscope.

DNA fragmentation assay

1×10^6 cells were treated with curcumin and genistein at the IC_{50} values for 48 h. Cellular DNA of treated cells was extracted from the cells according to Gong's modified method (Gong et al., 1994). Briefly, treated and untreated cells were trypsinized with 0.25% trypsin and collected by centrifugation (200 *g*, 10 min), washed twice in cold PBS (10 mM) and resuspended in hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, pH 7.4) containing 0.5% Triton X-100 for 30 min at 4 °C. The lysates were centrifuged at 13,000 *g* for 15 min at 4 °C. DNA was extracted from the supernatant with equal volume of phenol-chloroform-isoamylalcohol, precipitated by addition of two volumes of absolute ethanol and 0.1 volume of 3 mM sodium acetate and treated with RNase A (500 U/ml) at 37 °C for 3 h. The pattern of fragmentation was analysed on 1.5% agarose gel.

Methylation-specific PCR (MSP)

The effect of curcumin, genistein and allyl sulphide on the promoter hypermethylation of the *RARβ2* gene in the SiHa cell line was monitored by MSP (Lee et al., 2005) after treating the cells with natural compounds at 20 µM concentration for different time intervals, i.e. 48, 72 h and six days. The HeLa cell line was exposed to 20 µM curcumin for six days before conducting MSP for the *RARβ2* gene. DNA extracted from the cell lines (Hoque et al., 2004) was modified with sodium bisulphite and MSP was carried out (Herman et al., 1996) using specific primers for methylation and unmethylation for the *RARβ2* gene (Table 1).The annealing temperature used for MSP was 62 °C. 5-aza-2-deoxycytidine, a known demethylating agent, was used as a positive control.

Table 1. Primer Sequence for MSP

Gene	Primer Sequence for MSP	Annealing (°C)	Cycles	Product size (bp)
RARβ2	M Forward: 5'- TCGAGAACGCGAGCGATTCG -3' M Reverse: 5'- GACCAATCCAACCGAAACGA -3' U Forward: 5'- TTGAGAATGTGAGTGATTTGA -3' U Reverse: 5'- AACCAATCCAACCAAAACAA -3'	62 °C	35	146

Table 2. Primer Sequence for RT-PCR

Gene	Primer Sequence for RT-PCR	Annealing (°C)	Cycles	Product size (bp)
RARβ2	Forward:5'- GACTGTATGGATGTTCTGTCAG-3' Reverse: 5'- ATTTGTCCTGGCAGACGAAGCA-3'	61 °C	35	256
β actin	Forward: 5'- GTGGGCCGCTCTAGGCACCA- 3' Reverse: 5'- GGTTGGCCTTAGGGTTCAGGGGGG- 3'	61 °C	35	245

RT-PCR

RNA was isolated from the treated and untreated SiHa and HeLa cells after six days using trizol reagent. An equal amount of RNA was used to synthesize cDNA using the RevertAid first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). RT-PCR was carried out to check the alteration in the level of mRNA expression after the treatment with curcumin, genistein and 5-aza-2-deoxycytidine using primers as given in Table 2. β actin was used as the internal control.

Results

The IC_{50} values of curcumin and genistein in SiHa cells were found to be nearly 50 and 80 μM, respectively, by the MTT assay. In case of allyl sulphide, no cytotoxicity was found as the optical density values were more or less similar to the control until 1000 μM concentration. Hence, allyl sulphide treatment was given at 50 μM concentration. The IC_{50} value of curcumin was observed to be 50 μM in HeLa cells (Fig. 1).

The morphological studies and ethidium bromide staining showed that curcumin and genistein treatment resulted in formation of apoptotic bodies in SiHa cells. Allyl sulphide treatment didn't show any characteristics of apoptosis (Fig. 2, Fig. 3). Similarly, the morphological studies in HeLa cells also showed formation of apoptotic bodies after the treatment with curcumin (Fig. 2). Internucleosomal DNA fragments were observed only in curcumin- and genistein-treated SiHa cells. Allyl sulphide treatment was unable to produce any fragment. Internucleosomal fragments were also observed in curcumin-treated HeLa cells (Fig. 4).

Treatment with 20 μM of genistein or curcumin resulted in reversal of the methylation status of the *RARβ2* gene after 72 h (Fig. 5A) and this demethylation was observed to increase as the time period of treatment was increased to six days (Fig. 5B). This was corroborated by the appearance of unmethylation-specific band (UMSB) after the treatment with the test compound for six days (Fig. 5C). Genistein treatment resulted in a higher level of mRNA expression as compared to curcumin in SiHa cells (Fig. 6). The result obtained with SiHa cells was similar to the result obtained in the HeLa cell line, which showed the reversal of hypermethylation after the treatment with 20 μM of curcumin (Fig. 7).

Discussion

Much of the contemporary research is focused on the study of epigenetic changes resulting in many types of neoplasia and their possible reversal using natural compounds since, unlike genetic changes, the epigenetic changes such as DNA methylation can be reversed.

MSP was carried out using methylation-specific primers to check the ability of natural compounds to cause reversal of hypermethylation (Herman et al., 1996).

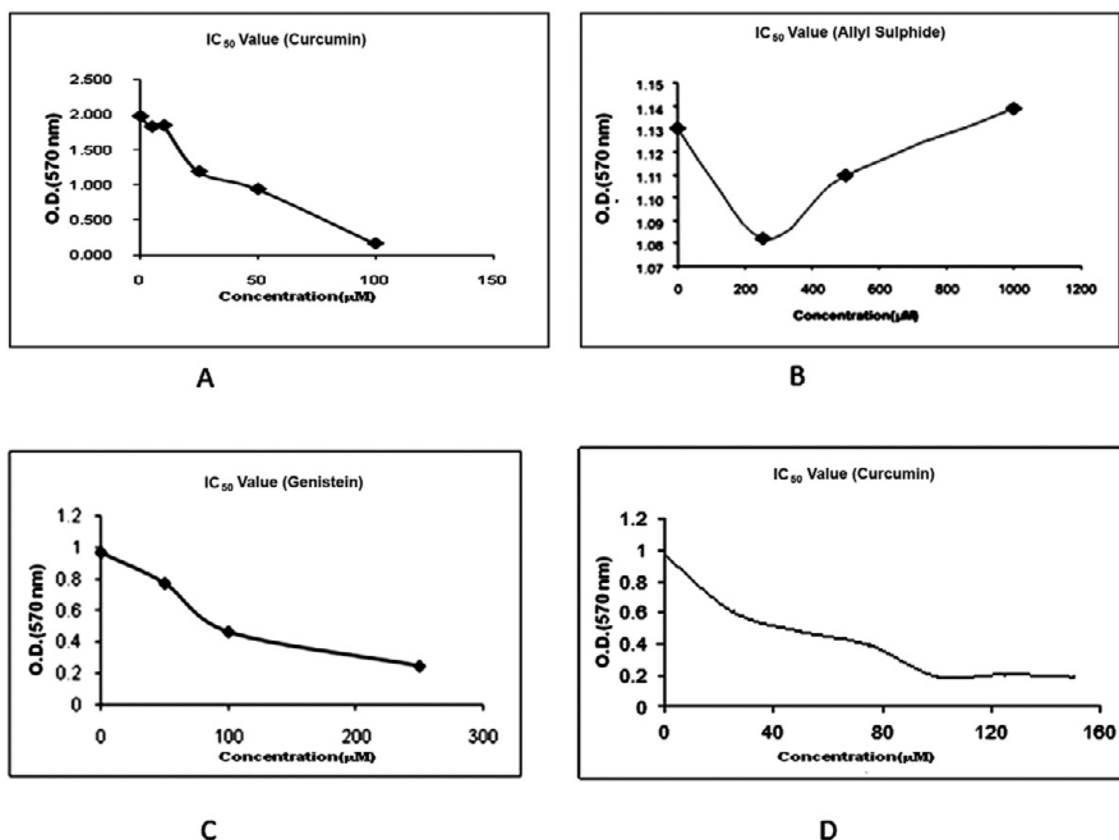

Fig. 1. MTT assay to estimate cell viability after the treatment of SiHa cells with (A) curcumin, (B) allyl sulphide, and (C) genistein for 48 h. (D) MTT assay after treatment of HeLa cells with curcumin for 48 h

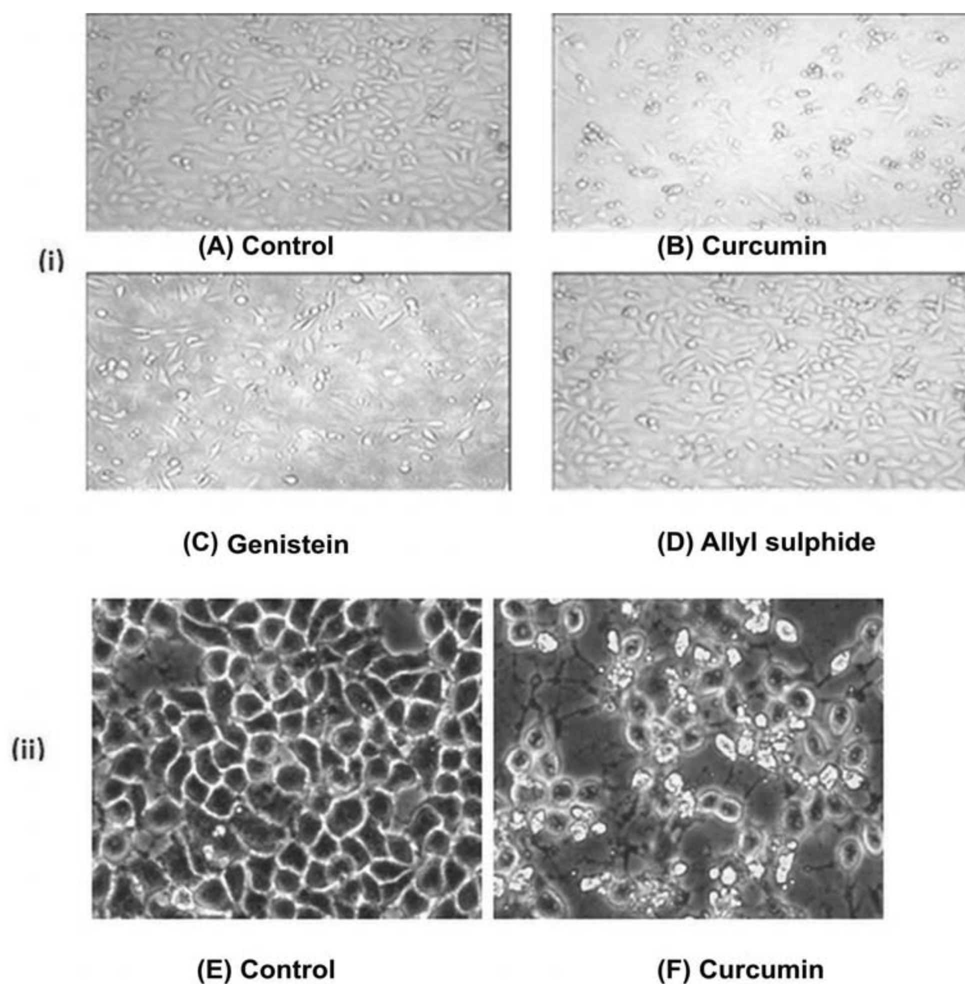

Fig. 2. (i) Morphological changes observed in SiHa cells after 24 h. (A) control, (B) curcumin treatment, (C) genistein treatment at IC_{50} values, and (D) allyl sulphide (50 μM) treatment under a phase-contrast microscope (10×)
(ii) Morphological changes observed in HeLa cells after 24 h. (E) control, (F) curcumin treatment at IC_{50} value

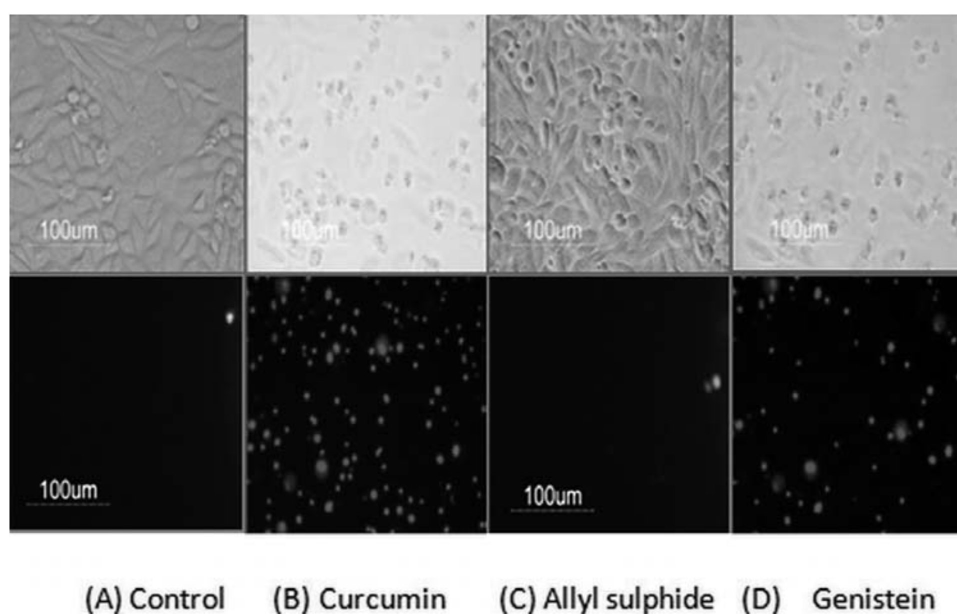

Fig. 3. Ethidium bromide (100 μg/ml) staining of SiHa cells. (A) control, (B) curcumin treatment, (C) allyl sulphide treatment, (D) genistein treatment for 96 h (40×). The treatments were carried out at IC_{50} values except in case of allyl sulphide (50 μM)

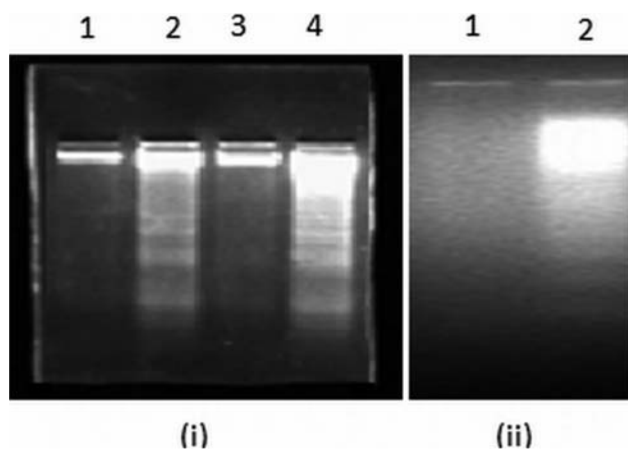

Fig 4. (i) Induction of apoptosis in SiHa cells. (1) control, (2) genistein (80 µM), (3) allyl sulphide (50 µM), (4) curcumin (50 µM) for 48 h
(ii) Induction of apoptosis in HeLa cells. (1) control, (2) curcumin (50 µM) for 48 h

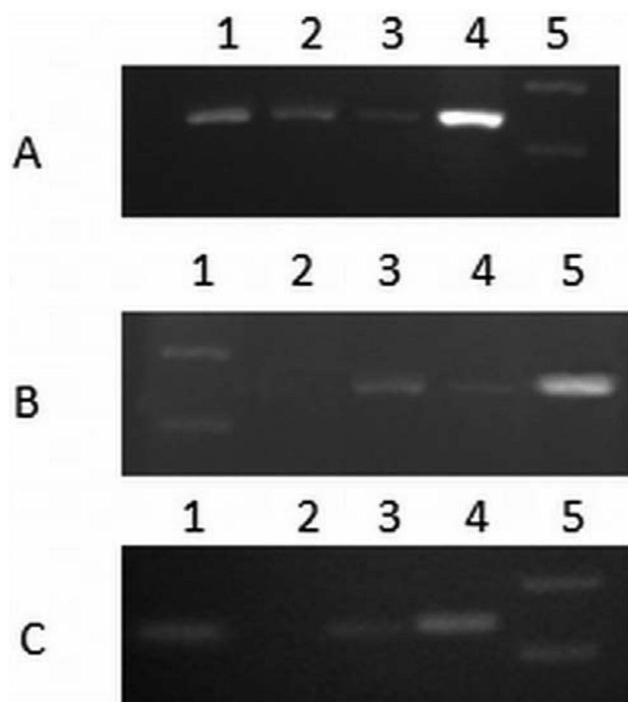

Fig 5. (A) MSB (methylation-specific band) of the *RARβ2* gene in the SiHa cell line after 72 h of treatment with (1) curcumin, (2) genistein, (3) 5-aza-2-deoxycytidine, (4) control (without treatment), (5) 100 bp ladder
(B) MSB of the *RARβ2* gene in the SiHa cell line after 6 days of treatment with curcumin and genistein. Cells were treated with 20 µM of curcumin, genistein and 5-aza-2-deoxycytidine for 6 days. (1) 100 bp ladder, (2) 5-aza-2-deoxycytidine, (3) curcumin, (4) genistein, (5) control
(C) UMSB (unmethylation-specific band) of the *RARβ2* gene in the SiHa cell line after 6 days of treatment. (1) genistein, (2) control, (3) curcumin, (4) 5-aza-2-deoxycytidine (positive control), (5)100 bp ladder

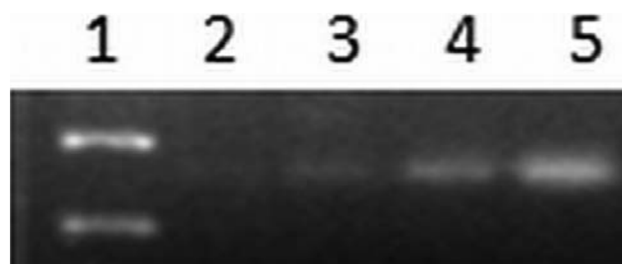

Fig. 6. Alterations in levels of mRNA expression of the *RARβ2* gene in the SiHa cell line after 6 days of treatment with curcumin, genistein and 5-aza-2-deoxycytidine. mRNA expression was determined using RT-PCR. (1) 100 bp ladder, (2) control, (3) curcumin, (4) genistein, (5) 5-aza-2--deoxycytidine

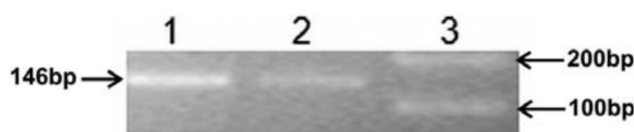

Fig. 7. Methylation-specific band of the *RARβ2* gene in the HeLa cell line after 6 days of treatment with 20 µM of curcumin. (1) control, (2) curcumin, (3) 100 bp ladder

After calculating the IC_{50} values for the compounds, the treatment was given at 20 µM as recent reports have shown the demethylating activity of EGCG and genistein at this concentration in some oesophageal and prostate cancer cell lines (Fang et al., 2003; 2007). Tea polyphenols (catechin, epicatechin, and (-)-EGCG) and bioflavonoids (quercetin, fisetin and myricetin) inhibit SssI DNA methyltransferase (DNMT) and DNMT1-mediated DNA methylation in a concentration-dependent manner (Lee et al., 2005). Caffeic acid and chlorogenic acid have been shown to partially inhibit methylation of the promoter region of the *RARβ* gene (Lee et al., 2005).

The demethylating ability of the dietary components such as genistein and lycopene has been shown to have varied behaviour in different cell lines and genes. The *RARβ2* gene was not demethylated by genistein or lycopene in both MCF-7 and MDA-MB-468, which are breast cancer cell lines. However, lycopene did induce demethylation of *RARβ2* and *HIN1* genes in the non-cancer MCF10A fibrocystic breast cells (King-Batoon et al., 2008).

Curcumin has been shown to inhibit HPV infection in cervical cancer (Prusty and Das, 2005). Molecular docking of the interaction of curcumin and DNMT1 suggests that curcumin covalently blocks the catalytic thiolate of C1226 of DNMT1 to exert its inhibitory effect. Curcumin and one of its major metabolites, tetrahydrocurcumin can inhibit M. SssI, a DNMT1 analogue, activity (Zhongfa et al., 2009). According to a recent study, several phytochemicals inhibit the DNA methyltransferase activity, with betanin being the weakest while rosmarinic and ellagic acids the most potent modulators (up to

88% inhibition) among the compounds selected for the study (Paluszczak et al., 2010).

This is the first report on the ability of genistein and curcumin to cause the reversal of hypermethylation and reactivation of the *RARβ2* gene in a cervical cancer cell line. It was observed that after six days of treatment genistein and curcumin treatment resulted in significant inhibition of promoter hypermethylation in the SiHa cell line. The methylation-specific band (MSB) showed a time-dependent decrease in intensity after the treatment with genistein and curcumin. The expression of mRNA increased approximately proportionally to the appearance of unmethylated DNA, whereas PCR products from methylated DNA decreased as the time period of treatment was increased. However, genistein was more effective in causing the reversal of promoter hypermethylation.

Further work is being carried out to determine the minimum effective concentration because genistein and lycopene have been found to be effective even at low concentrations such as 2 μM (King-Batoon et al., 2008), as well as the concentration-dependent change after the treatment with these compounds.

The reversal of hypermethylation and re-expression of the *RARβ2* gene by genistein and curcumin was similar in pattern but lower than that produced by the classic DNA methyltransferase inhibitor 5-aza-2-deoxycytidine. Genistein and curcumin act as double-pronged agents causing apoptosis as well as reversal of promoter hypermethylation of the *RARβ2* gene. These natural compounds are an important component of our diet and hence do not have any cytotoxic effects on normal cells unlike the demethylating chemicals. The reversal of these epigenetic changes by natural compounds could prove to be significant in the direction of therapy of cancer.

Acknowledgements

The authors acknowledge the help provided by Dr. Jaswant Singh and Mr. Ajay Mahajan during the apoptotic studies on the HeLa cell line. The help provided by Ms. Neetu Bharti during the cell culture studies on the SiHa cell line is also acknowledged.

References

Angeles, E., Vázquez-Valadez, V. H., Vázquez-Valadez, O., Velázquez-Sánchez, A. M., Ramírez, A., Martínez, L., Díaz-Barriga, S., Romero-Rojas, A., Cabrera, G., López-Castañares, R., Dueñas-González, A. (2005) Computational studies of 1-hydrazinophtalazine (hydralazine) as antineoplastic agent. Docking studies on methyltransferase. *Lett. Drug Des. Discov.* **4,** 282-286.

Dorai, T., Aggarwal, B. (2004) Role of chemopreventive agents in cancer therapy. *Cancer Lett.* **215**, 129-40.

Fang, M. Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, Y., Welsh, W., Yang, C. S. (2003) Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res.* **63**, 7563-7570.

Fang, M., Chen, D., Yang, C. S. (2007) Dietary polyphenols may affect DNA methylation. *J. Nutr.* **137,** 223S-228S.

Freshney, R. I. (1994) *Culture of Animal Cells. A Manual of Basic Techniques,* Third Edition. John Wiley & Sons, Inc. New York, NY.

Gong, J., Traganos, F., Darzynkiewicz, Z. (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218,** 314-319.

Gonzalez, A. D., Lizano, M., Candelaria, M., Cetina, M. L., Arce, C., Cervera, E. (2005) Epigenetics of cervical cancer. An overview and therapeutic perspectives. *Mol. Cancer* **4,** 38.

Heckenkamp, J., Leszczynski, D., Schiereck, J., Kung, J., La Muraglia, G. M. (1999) Different effects of photodynamic therapy and gamma irradiation on vascular smooth muscle cells and matrix: implications for inhibiting restenosis. *Arterioscler. Thomb. Vasc. Biol.* **19,** 2154-2161.

Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., Baylin, S. B. (1996) Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands, *Proc. Natl. Acad. Sci. USA* **93**, 9821-9826.

Hoque, M. O., Begum, S., Topaloglu, O., Jeronimo, C., Mambo, E., Westra, W. H., Califano, J. A., Sidransky, D. (2004) Quantitative detection of promoter hypermethylation of multiple genes in the tumor, urine, and serum DNA of patients with renal cancer. *Cancer Res*. **64,** 5511-5517.

King-Batoon, A., Lesczynska, J. M., Klein, C. B. (2008) Modulation of gene methylation by genistein or lycopene in breast cancer cells. *Environ. Mol. Mutagen.* **49**, 36-45.

Lee, W. J., Shim, J. Y., Zhu, B. T. (2005) Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. *Mol. Pharmacol.* **68**, 1018-1030.

Paluszczak, J., Krajka-Kuzniak, V., Baer-Dubowska, W. B. (2010) The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells. *Toxicol. Lett*. **192**, 119–125.

Prusty, B., Das, B. (2005) Constitutive activation of transcription factor AP-1 in cervical cancer and suppression of HPV transcription and AP-1 activity in HeLa cells by curcumin. *Int. J. Cancer* **113,** 951-960.

Zhongfa, L., Zhiliang, X., William, J., Ryan, E. P., Shujun, L., Jianhua, Y., Pui-kai, L., Jiayuh, L., Jame, R. F., Marcucci, G. M., Chenglong, L., Chan, K. K. (2009) Curcumin is a potent DNA hypomethylation agent. *Bioorg. Med. Chem. Lett*. **19,** 706–709.