International Journal of Health Sciences and Research
www.ijhsr.org ISSN: 2249-9571

Original Research Article

Demethylation of RASSF1A Gene by Quercetin and Eugenol in HeLa Cancer Cell Line

Saloni[1], Archita Sharma[1], Harsh Goel[1], Sweta Pal[1], Pooja Rai[1], Kavita Rawat[2], Saima Syeda[2], Anju Shrivastava[2], Abhimanyu Kumar Jha[1]

[1]Department of Biotechnology, Institute of Applied Medicines and Research Ghaziabad, Uttar Pradesh, India
[2]Department of Zoology, Delhi University, India

Corresponding Author: Abhimanyu Kumar Jha

ABSTRACT

Cervical cancer is a potentially preventable disease and second most familiar malignancy among women worldwide with high incidence rates in developing regions. A worldwide approximated annual case of cervical cancer is 493,000 and ends with 273,500 every year deaths. In developing countries, it reports for about 85% of both its morbidity and fatality. Epigenetic silencing of neoplasm inhibitor genes may be reversed by numerous molecules additionally as natural compounds like polyphenols which are able to act as a demethylating agent. Quercetin and Eugenol have been found to specifically target numerous neoplasm inhibitor genes and modify their expression. To analyse the response of quercetin and eugenol on the methylation pattern of the RASSF1A gene in HeLa neoplastic cell line in a dose-dependent manner. The reversal of hypermethylation of the RASSF1A gene was observed after 6 days of treatment with 20 µM Quercetin and Eugenol in HeLa cells. To analyse, the reversal of methylation imitate of hypermethylated RASSF1A gene in HeLa cervical cancer cell line, it was treated with various concentrations of Quercetin and Eugenol for various time periods. Our results demonstrate that treatment of Cervical cells with Quercetin and Eugenol reversed the hypermethylation status of the RASSF1A gene.

Keywords: Cervical cancer, HeLa cell line, Quercetin, Eugenol, Hypermethylation

INTRODUCTION

Cervical cancer is the third common cancer among women; around 70% of the patients are infected by HPV (16/18) virus (Torre et al., 2015). Unfortunately, this neoplasia is one of the foremost common life-threatening diseases within the world and also the patients sometimes had but 5 years of survival time after diagnosis. Although combined treatment of chemotherapy, surgery and radiation are recommended for this cancer, still it had a poor prognosis (Siegel et al., 2016). It had been demonstrated the role of epigenetic and genetic factors in tumorigenesis. Epigenetic events show phenotype alterations without genetic alterations. DNA hypermethylation and histone modification are two important epigenetic events. DNA hypermethylation in CpG Islands of the promoter region in neoplasm inhibitor genes induces gene suppressing without alteration in DNA sequences which are inherited from one generation to the consequent (Ujihira et al.,2016). The Ras

association domain family 1 isoform A gene expresses a RASSF1 family macromolecule involved in cell cycle suppression, programmed cell death, and genetic instability. It shows a key act in inhibiting Ras-mediated oncogenesis. Promoter hypermethylation is the most common mechanism for transcriptional suppressing of the gene and has been constructed in numerous epithelial neoplasm (Donninger etal.,2016)

As synthetic compounds could have cytotoxic effects, the focus is on natural products for the epigenetic reversal. Phytochemicals are biologically progressive compounds organize in plants that had defensive and disease-preventive possessions(Huang et al., 2010) Quercetin derived from quercetum (oak forest), after Quercus and active parts of the medicative herbs like St John's wort (St. Johns Wort) and black elder L (Elderflower). In neoplasm cells, quercetin blocked cell cycle and elicited pro-apoptotic effects while not poignant traditional cells (Gibellini et al., 2011; Chirumbolo, 2013). Quercetin has anti-estrogenic effects and thus might cut back the pathological process potential of carcinoma cells. Eugenol isolated from varied sources including *Syzygium aromaticum* and *Ocimum sanctum*. Eugenol, a methoxy phenol element of clove (*Syzygium aromaticum*, Family Myrtaceae), has been accorded variety of medical speciality effects, together with the inhibitor, anti-inflammatory, analgesic, anaesthetic, antipyretic, antiplatelet, antianaphylactic, medication, an antiepileptic drug, antihyperglycemic, bactericide, antifungal and antiviral effects (Thompson et al.,1998)

HeLa cell line

The HeLa cell line derived from the cervical cancer cells of Henrietta Lacks, a 31-year old female patient, in 1951 who expired of the tumor on October 4, 1951 The HeLa cell line is remarkable in that it is the primary human cell line able to survive in vitro indefinitely (Hirchaud et al.,2013). HeLa cells were taken from a tissue pattern from the adenocarcinoma, which has a modal chromosome number of 82. The cells had an active type of telomerase during cell division, and as a result, the cell line propagates extremely rapidly in corresponding to other cell lines (Rahbari et al., 2009). A recent estimate is a "hypertriploid chromosome number (3n+)" which implies 76 -80 total chromosome with 22–25 clonally abnormal chromosomes, referred as HeLa signature chromosomes (Nakano et al.1998)."Human papillomaviruses (HPVs) are generally aggregated into the cellular DNA in cervical cancers. The HPV18 type HeLa cells are widely studied carcinoma line revealing low-level wild type p53.On the other hand, the origin of HPV16-positive lines CaSki and SiHa is squamous cell carcinoma. Adenocarcinoma and squamous cell carcinoma are the two main types of cervical cancers. Whereas adenocarcinomas of the uterine cervix represent only 15- 20% of all primary cervical carcinomas their incidence has grown progressively throughout the past decades (Jones et al.,2006).

MATERIALS AND METHODS

Collection of cell line

The Cervical cancer cell line, HeLa was purchased from the National Centre for Cell Sciences (Pune, India). Quercetin and Eugenol were procured from HiMedia (Mumbai, India).

Culture of cell lines

HeLa cell line was cultured according to standard protocols (Freshney, 1994). The cells were cultured in

supplemented with 10% heat-inactivated FBS in 5% CO_2 at 37 ℃. The cells were re-supplemented with fresh medium and test compounds every 48 hour.

Cytotoxicity of chemopreventive agents

The cytotoxicity of quercetin and eugenol was studied on the cervical cancer cell lines by the cell proliferation assay. This assay was carried out to estimate cell viability after treatment with the test compounds.$5x10^4$ cells were seeded in 96 well culture as per the need of doses and checkpoints. The incubated cells were kept in 200µl/ml DMEM for 2hrs 30 minutes for adherence. All the wells were treated with the specific chemicals like PBS and methanol beside control and incubated for respective time periods.

Crystal violet assay

25µl of crystal violet was added to all the wells containing the treatment. 10µl of 1% SDS was added to each of the wells to solubilize the stain. The wavelength was set to 570nm and the appropriate readings were taken. These readings were noted down and specific graphs were formulated. The graph made from the readings gave the IC_{50} values of the Quercetin and Eugenol. Based on these values, efficient doses of treatment were formulated.

DNA isolation

Cells obtained after the cell culture process were lysed in digestion buffer (10µM Tris-HCl, pH 7.6, 0.5Mm EDTA, pH 8.0 and 20% SDS containing proteinase K (20µL).DNA was then purified using standard PCI (phenol-chloroform-isoamyl alcohol) extraction and the final treatment of chilled ethanol was given.

Sodium Bisulphite modification

Bisulfite modification was done using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irwin, USA. Sodium bisulphite of the cell line was done. (130µl CT conversion reagent, 20µl DNA)

PCR reaction was then carried out in a thermal cycler following two cycles:

1. 98°C for 10 min
2. 64°C for 2.5 hours

Then the addition of specific buffers was done (600µl M-binding buffer, 100µl M-wash buffer. 200µl D-sulphonation buffer and 10µl Elution buffer. Methylation specific PCR (MS-PCR)

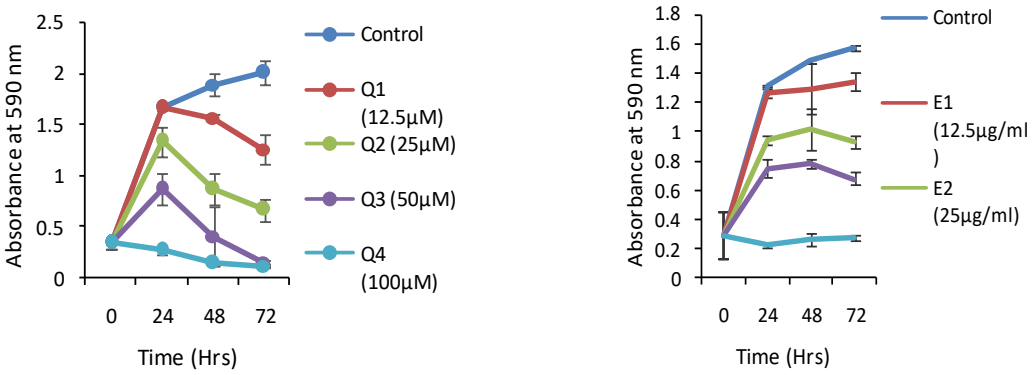
Table1: Primer sequence for MSP

Gene	Primer Sequence for MSP	Annealing Cycles	Product (bp)	size (°C)
RASSF1A	M Forward- 5'-GGGTTTTGCGAGAGCGCG-3'	62 ℃	35	146
	M Reverse: 5'-GCTAACAAAGCGGAACCG-3'			
	U Forward: 5'-GGTTTTGTGAGAGTGTGTTTAG-3''			
	U Reverse: 5'-CACTAACAAACACAAACCAAAC-3''			

DNA isolated from the cell lines was modified with sodium bisulphite and MS-PCR was carried out using specific primers for methylation and unmethylation for the RASSF1Agene (Table1.) The amplified products were further run on a 2% agarose gel.

RESULTS

The IC_{50} values of quercetin and eugenol in HeLa cells were found to be nearly 50 and 80 µM, respectively, by the crystal violet assay. Hence, quercetin and eugenol treatment was given at 20 µM concentration. The IC_{50} value of quercetin was observed to be 48.3µg/mol in HeLa cells while IC_{50} value of eugenol was 46.59 µg/ml in HeLa cells (Figure 1)



(a)Quercetin (IC_{50} = *48.3µg/mol*) **(b) Eugenol (IC_{50} = *46.59 µg/ml)***

Fig.1 Treatment of HeLa with phytochemicals under different time periods (24, 48 and 72hours) (% viability of HeLa cell line on treatment with phytochemical)

The HeLa cells were treated with different concentrations of the quercetin and eugenol compound and viewed under phase-contrast microscope to visualize morphological changes. But at higher concentration of 100µM, there was more growth inhibition with increased amount of cell deaths (Figure 2&3).

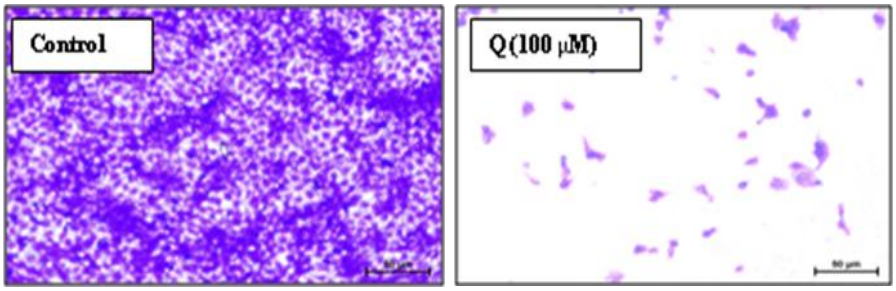


Fig.2 Morphological changes observed in HeLa cell line after treatment with *Quercetin* (6days treatment)

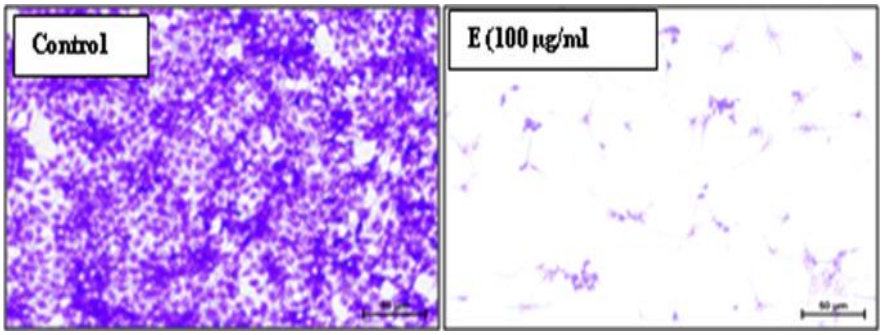


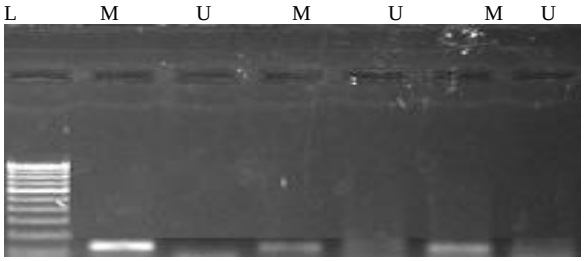
Fig.3 Morphological changes observed in HeLa cell line after treatment with *Eugenol* (6days treatment)

Crystal violet assay reveals the apoptosis which took place in HeLa cell line in different doses of treatment of drugs. Since methylation specific band (MSB) was not observed in HeLa cell line, but the unmethylation specific band (UMSB) was clearly seen in HeLa cells. So therefore, treatment of the two natural compound quercetin and eugenol was done and still the unmethylation specific bands were observed.

DISCUSSION

Epigenetic gene suppressing via dense DNA methylation among CpG islands has been demonstrated to occur in several neoplasm types including human papillomavirus (HPV) associated with cervical cancer. Tumour suppressor genes

(TSGs) that are of clear importance in the pathogenesis of cervical cancer are common targets for gene silencing during this disease. In this study, we evaluated the apoptotic effect of RASSF1A in Hela cancer cell line. Reversal of the methylated DNA of cancer cell lines can also be shown by numerous phytochemicals (Fang et al., 2003). When the HeLa cells were treated with the Quercetin and Eugenol, reversal of hypermethylation was observed in cells after 6 days treatment. The DNA was isolated from the treated and untreated cells after treatment with these natural compounds for various time intervals (24 h, 48 h, 72 h and 6 days). This was followed by sodium bisulphite modification. After carrying out MSP, it was observed that methylation specific band was decreased in HeLa cells, but the unmethylation specific band was clearly increased in HeLa cells after 6 days of treatment with these natural compounds.



Ladder control control quercetin eugenol eugenol quercetin
Fig. 1: Methylation Specific Bands and Unmethylation Specific Bands after the treatment of 6days with 20 µM of Quercetin and 20 µM of Eugenol
L: Ladder
U: Unmethylation specific band
M: Methylation specific band

The present study focused to estimate the effect of Quercetin and Eugenol, on promoter hypermethylation of RASSF1A gene .The methylation specific bands was observed even after the treatment with these compounds but the intensity of these bands were decreased. It needs to be further checked whether it leads to the reactivation of tumor suppressor, RASSF1A which had been silenced in the selected cell line due to promoter hypermethylation. These findings may provide the approach for future development as cancer preventive agents.

CONCLUSION

Quercetin and Eugenol both compounds showed partial demethylation at the concentration of 20 µM after 6 days treatment while no epigenetic reversal was found till 72h. The reversal of these epigenetic changes by natural compounds could prove to be significant in the direction of therapy of cancer.

ACKNOWLEDGEMENT

The authors acknowledge the help provided during the study on "Demethylation of RASSF1A Gene by Quercetin and Eugenol in HeLa Cancer Cell Line" at Department of Biotechnology, Institute of Applied Medicines and Research.

REFERENCES

- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet- Tieulent J, Jemal A. Global cancer statistics. CA Cancer J Clin. 2012 ;65(2):87-108..
- Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin.2016; 66(1) :7-30.
- Ujihira T, Ota T, Kusunoki S, Sugimori Y, Kimura M, Kaneda H et al. Outcome of neoadjuvant intra-arterial chemotherapy and radical hysterectomy for treatment of bulky stage IB to stage IIB uterine cervical cancer: can postoperative irradiation be avoided? Int J Gynecol Cancer. 2016: 26(7);1258-63.
- Donninger H, Clark J, Rinaldo F, Nelson N, et al. The RASSF1A tumor suppressor regulates XPA-mediated DNA repair.Mol Cell Biol. 2015;35(1):277-87.
- Huang WY, Cai YZ, Zhang Y. Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. Nutr Cancer. 2010;62(1):1–20.
- Gibellini, L., Pinti, M., Nasi, M., Montagna, J. P., De Biasi, S., Roat, E., et al. .Quercetin and cancer chemoprevention. Evid. Based Complement. Alternat. Med. 2011 :59-136.
- Chirumbolo, S.Quercetin in cancer prevention and therapy. Integr. Cancer Ther. 2013; 12: 97–102

- Thompson, D. C.; Barhoumi, R.; Burghardt, R. C. "Comparative toxicity of eugenol and its quinone methide metabolite in cultured liver cells using kinetic fluorescence bioassays". Toxicology and Applied Pharmacology° 1998;149(1),55–63
- Hirchaud F, Hermetet F, Ablise M, et al. Isoliquiritigenin induces caspase-dependent apoptosis via downregulation of HPV16 E6 expression in cervical cancer CaSki cells. Planta Med. 2013;79:1628-35.
- Rahbari, R., Sheahan, T., Modes, V., Collier, P., Macfarlane, C., & Badge, R. M. (. A novel L1 retrotransposon marker for HeLa cell line identification. Biotechniques. 2009;46(4):277-284.
- Nakano K, Watney E, McDougall J.K. Telomerase activity and expression of telomerase RNA component and telomerase catalytic subunit gene in cervical cancer. Am. J. Pathol. 1998;153: 857–868.
- Jones PA ,Yoo CB, Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov. 2006 ;5(1):37–50.
- Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS,Tea polyphenol(_)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation silenced genes in cancer cell lines. Cancer Res 2013:63(22):7563–7570

How to cite this article: Saloni, Sharma A, Goel H et.al. Demethylation of RASSF1A gene by quercetin and eugenol in HeLa cancer cell line. Int J Health Sci Res. 2019; 9(8):29-34.

******