Geraniol Inhibits Endometrial Carcinoma via Downregulating Oncogenes and Upregulating Tumour Suppressor Genes

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Abstract Endometrial carcinoma is the fourth most abundant cancer worldwide in women. Female Wistar rats were segregated into five groups: group I–control, group II–MNNG (N-methyl-N′-nitro-N-nitrosoguanidine-150 mg/kg) administered through intravaginal detention of cotton absorbent, group III–geraniol (GOH) only, group IV–GOH-pretreated (7 days before the start of MNNG administration); and group V–Co-administration of geraniol with MNNG. In this study, reverse transcriptase-PCR of K-ras, MAPK, PI3K, Wnt/ β -catenin genes, TGF- β and expressions of PCNA, PTEN, progesterone receptor and E-cadherin by Western blotting were performed from endometrial cancer tissue and control tissues. The mRNA expressions of K-ras, MAPK, PI3K, Wnt/ β -catenin and TGF- β were amplified in MNNG induced group. Oral administration of GOH (both pre and co-administration) reversed the mRNA expression towards normal. The reversibility is more predominant in pretreatment groups ($p < 0.05$). The expression of PCNA was upregulated and downregulation of PTEN, progesterone receptor and E-cadherin was noticed in MNNG induced rats. Pre and co-administration of GOH significantly reversed the expression pattern of proteins. GOH treatment is more effective in pretreatment groups ($p < 0.05$). These results provide powerful evidences that GOH could influence modulation of MAPK pathways and Wnt signalling pathways in the prevention of endometrial carcinoma in rats.

Keywords Endometrial carcinoma · N-methyl-N′-nitro-N-nitrosoguanidine · Mitogen activated protein kinase · Proliferating cell nuclear antigen · Phosphotensin homolog gene

Introduction

Endometrial carcinoma (EC) is the frequently detected cancer in women worldwide [1]. EC is commonly spotted in female genital tract. Proteins that have been considered as biomarkers of endometrial cancer include K-ras, MAPK, PI3K, Wnt/ β -catenin and TGF- β which have predominant roles in signal transduction pathways causing growth and differentiation in endometrial cancer [2]. MAPK pathway is an alternative intracellular signaling cascade, which diffuses signals by phosphorylation on tyrosine kinase-type receptors in cell membranes. An independent and constitutive induction of MAPK signaling is often activated by the overexpression of growth factors and their receptors during carcinogenesis. The phosphatidylinositol 3-kinase (PI3K) pathways interacts bidirectionally with the Ras/mitogen-activated protein kinase (MAPK) pathway and the two pathways co-ordinate and regulate functional outcomes. PI3K pathway that signals downstream from receptor tyrosine kinases (RTK) is often activated in many cancer ancestries, including EC, by anomalies at multiple nodes [3]. The Wnt signaling pathway has been investigated in cancers, such as endometrial carcinoma and this pathway mediates several physiological actions in embryogenesis and adult homeostasis comprising cell cycle regulation, control of proliferation, migration and in normal follicular development [3]. The Wnt/ β -catenin accumulates and translocates into the nucleus where it binds with T cell factor/lymphoid enhancer factor resulting

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activation of gene mutation in ovarian cancer [4]. Activated β -catenin inhibits E-cadherin expression. E-cadherin is a transmembrane protein that regulates cell–cell adhesion and during carcinogenesis, and it is not expressed in tumor tissues. TGF- β is an effective tumor suppressor and is recognized on the control of cellular responses such as cell growth, recognition, apoptosis, differentiation and cell fate specification during embryogenesis. In EC, disrupted expression of TGF- β was noticed at mRNA and protein levels, implicating prolonged cell survival and tumor progression [5].

Phosphatase homology (PTEN) is a tumour suppressor gene and is intermittently deleted or mutated in numerous types of human cancers, such as, melanoma, glioma, prostate and endometrial carcinoma [6]. Most often in glioma and prostate tumor, a higher rate of mutations is reported which indicate that PTEN may play a role in tumor progression and decreased PTEN expression may accompany the progression of ovarian cancer [6]. Proliferating cell nuclear antigen (PCNA) is a homotrimeric protein and is expressed in the nuclei of cells during S phase of cell cycle. Increased expression of PCNA is a known diagnostic marker for endometrial hyperplasia and endometriosis [7]. Progesterone is imperative for regulation of usual reproductive function and plays key roles in controlling changes in the uterus and ovaries during menstrual cycle. The influence of progesterone is mediated via progesterone receptor (PR) and during endometrial carcinogenesis, progesterone receptor expression is differentially down regulated [8].

The overall incidence of EC has been increased and the onset of endometrial carcinoma has been substantially enhanced in animals receiving an intra-uterine administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) through the vagina [9]. MNNG has been used as a potent mutagen/carcinogen; it is a typical DNA damaging agent that inserts methyl groups to several nucleophilic sites of DNA bases, finally causing DNA double-strand breaks [9]. MNNG is also known to methylate proteins and could induce adenocarcinomas in a wide range of mammals. Although MNNG has been employed to unravel pathways of DNA damage and repair the intricacies of DNA damage mediated signaling pathways are to be understood entirely [10].

Geraniol (GOH) an acyclic monoterpene alcohol normally isolated from *Cymbopogon flexuosus* [11], exerts both antitumor activity (in vitro and in vivo conditions) in opposition to different cancer types. GOH can modify signal transduction pathways involved in cellular proliferation and apoptosis by modulating the levels of redox sensitive transcription factors and enzymes like mitogen activated protein (MAP) kinases. The antitumor potential of GOH against murine leukemia, melanoma, breast and

colon cancer has been previously documented [12]. However, the potential effects of GOH against endometrial carcinogenesis have not been investigated. Therefore, a systematic investigation on the defensive role of GOH in preventing MNNG induced endometrial cancer by analyzing the expression of biomarkers by RT-PCR (K-ras, MAPK, PI3K, Wnt/ β -catenin and TGF- β) and western blotting (PCNA, PTEN, progesterone receptor and E-cadherin) techniques have been performed.

Methodology

Experimental Animal

Healthy adult female Wistar rats, bred and reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University were used for the experiments. Animals (180–200 g) were housed in polypropylene cages lined with husk as bed and maintained in light/dark conditions (12 h light/dark). The animals were allowed free access to water ad libitum and standard pellet diet (Hindustan Liver Ltd., Bangalore, India). Animal handling and experimental procedures were approved (approval No. 980, dated 07/02/2013) by the Institutional Animal Ethics Committee, Annamalai University, India.

Chemicals Purchased

Geraniol and MNNG were purchased from Sigma Aldrich, Saint Louis, MO, USA and antibodies such as PCNA, PTEN, progesterone receptor, E-cadherin were purchased from Santa Cruz Biotechnology, India.

Experimental Design

Animals were randomly segregated into five groups of six animals each. Group I (control) rats received food ad libitum, group II–MNNG (150 mg/kg, dissolved in 0.2 ml olive oil) given by intravaginal detention of cotton absorbent twice a week for 90 days [13], group III–treated with geraniol (150 mg/kg b.wt) dissolved in 0.1 % corn oil [14], group IV–pre-treatment with GOH before 1 week of experiment and group V–co-administration of MNNG + GOH.

RNA Extraction and RT-PCR

Total RNA was isolated from tissue after homogenising 100 mg endometrial tissue using TRIzol reagent (Invitrogen Life Technologies, USA), and following the manufacturer's recommendations. The amount and purity of RNA extracted was analyzed by NanoDrop (ND-3300, NanoDrop

Technologies, USA) and agarose gel electrophoresis. By employing first-strand cDNA as a template, the specific primers K-ras (sense-5' CTGAAGATGTGCCTATGGTCC T 3' and antisense-5'GGAATCCCATAACTCCTTGC3'), PI3K (sense 5'TTAAGCCGGACCTCATTGAC 3' and antisense-5' GCAAATCCTCGTCATCGTCT3'), MAPK (sense-5'GCCTCTCCCATGCTTGTCTA3' and antisense-5'AGGTCACCAGGGTTTCACTG3'), wnt/ β -catenin (sense-5' GCCAGTGGATTCCGTACTGT3' and antisense-5'ATTT TCTGCAGCCCACCAG 3'), TGF- β (sense-5'CCCAC CCAGCAATCTATGAC 3' and antisense- 5'CGTGTTGC TCCACAGTTGAC 3') and GAPDH (sense-5'GCCAGGG ATGGAGAGAAGTA3' and antisense-5'ACTTGCGACCC TAAATACACC 3') (as reference gene) were subjected to 35 cycles of PCR amplification (30 s denaturation at 94 °C, 30 s annealing temperature consistent with the primer used and 2 min extension at 72 °C) in a thermal cycler (Bio-rad, Mini-cycler USA). The amplified products were determined by gel electrophoresis (Bio-rad submarine electrophoresis, USA) on 1.5 % agarose and visualized by ethidium bromide (0.5 % g/ml). Images of the RT-PCR ethidium bromide stained agarose gel were acquired using gel documentation system (Medi-Care, USA). The intensities of bands of above mentioned genes were normalized in relation to that of GAPDH bands by dividing the former by the GAPDH specific PCR product densities. GAPDH acted as control for sample to sample differences in reverse transcription and PCR conditions and the level of degradation and upturn of RNA [15].

Western Blot Analysis

After the experimental duration (90 days), tissue and blood samples were collected from all groups. The tissue was homogenised with radio immunoprecipitation assay (RIPA) buffer and the supernatant was collected. The protein concentration was quantified using bovine serum albumin as the standard.

The total protein was mixed with sample buffer and heated for 10 min. The sample mixture was run on 11.5 % sodium dodecyl sulphate (SDS-PAGE) gel for 1 h. The membrane was blocked in blocking buffer containing 5 % skimmed milk powder for 2 h. After 2 h, the blocked membranes were incubated with primary antibody (1:1000) for 6 h. After primary antibody incubation, the membranes were washed two times with blocking buffer for 15 min each. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (GENEI, Bangalore, India) was added. Subsequent to two intermittent washes with 1X TTBS and TBS, membranes were developed using 3,3'-diaminobenzidine (DAB) and visualized with the help of Chemi Doc Imaging System (Bio-Rad, USA) to detect signal. Then the membranes were stripped and re probed with mouse monoclonal anti β -actin

primary antibody (1:5000) (Santacruz Biotechnology, India). The values are measured by densitometric analyses of the blots which normalized to β -actin. The column heights are the mean $\pm$ SD of six determinants.

Statistical Analysis

The data of diverse biochemical parameters were examined by one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Values were deemed statistically significant when $p < 0.05$.

Results

The mRNA expressions of oncogene markers K-ras, MAPK, PI3K, wnt/ β -catenin and TGF- β were upregulated in MNNG induced rats as compared to GOH treated (pre and co-administration) rats and in rats treated with GOH only. The reversibility is more predominant in pretreatment groups ($p < 0.05$ (Fig. 1; Table 1). Noticeable increase in the expression of PCNA was found in MNNG induced endometrial cancer rats compared to control. Administration of GOH (pre and co-administered) to MNNG treated rats showed significantly decreased expression of PCNA. The protein expression of PTEN, progesterone receptor and E-cadherin was down regulated in MNNG induced rats compared to control. GOH treatment (pre and co-administration) to tumor bearing rats showed considerably up regulated PTEN, progesterone receptor and E-cadherin expression (Fig. 2; Table 2). The reversibility is more predominant in pretreatment groups ($p < 0.05$).

Fig. 1 Reverse transcriptase-PCR analysis of K-ras, PI3K, MAPK, Wnt/ β -catenin and TGF- β mRNA expression in control and experimental rats. Lane 1 Control, Lane 2 MNNG, Lane 3 GOH, Lane 4 GOH + MNNG (Pretreatment), Lane 5 MNNG + GOH (Co-administration)

Table 1 Reverse transcriptase-PCR analysis of K-ras, PI3K, MAPK, Wnt/ β -catenin and TGF- β mRNA expression in control and experimental rats

Protein markers	Control	MNNG	GOH	MNNG + GOH (Pre-treatment)	MNNG + GOH (Co-administration)
K-ras	1.76 $\pm$ 0.11	2.81 $\pm$ 0.16 [#]	2.23 $\pm$ 0.11	2.35 $\pm$ 0.16**	2.61 $\pm$ 0.13**
MAPK	1.46 $\pm$ 0.08	2.94 $\pm$ 0.18 [#]	2.31 $\pm$ 0.14	2.19 $\pm$ 0.11**	2.27 $\pm$ 0.11**
β -catenin	1.3 $\pm$ 0.06	2.22 $\pm$ 0.11 [#]	1.86 $\pm$ 0.1	1.71 $\pm$ 0.08**	1.58 $\pm$ 0.09**
PI3K	1.03 $\pm$ 0.46	1.81 $\pm$ 0.09 [#]	1.27 $\pm$ 0.06	1.4 $\pm$ 0.06**	1.69 $\pm$ 0.1**
TGF- β	1.07 $\pm$ 0.5	2.04 $\pm$ 0.1 [#]	1.37 $\pm$ 0.08	1.67 $\pm$ 0.08**	1.74 $\pm$ 0.11**

The mRNA expression of MNNG induced group was upregulated in K-ras, PI3K, MAPK, Wnt/ β -catenin and TGF- β . During GOH treatment (pre and Co-administration) K-ras, PI3K, MAPK, Wnt/ β -catenin and TGF- β expressions down regulation was noticed. The mean $\pm$ SD are indicated (SD as error bars). [#] (MNNG) significantly ($p < 0.05$) different from normal groups, ** (MNNG + GOH) significantly different from MNNG group

Fig. 2 Western blotting analysis of cell proliferation markers and components of signalling pathways during endometrial carcinogenesis. Lane 1 Control, Lane 2 MNNG, Lane 3 GOH, Lane 4 GOH + MNNG (Pretreatment), Lane 5 MNNG + GOH (Co-administration)

Discussion

In general K-ras mutations occur most abundantly in mucinous tumors, such as borderline malignancies than in non-mucinous cancer. K-ras mutation is more common in ovarian cancer than in other invasive cancers. MNNG could induce mutations by forming O⁶-methylguanine, subsequent mispairing with deoxythymidine during DNA replication, causing base substitutions from G $\rightarrow$ A. Further, GOH is known to cause GOH decline of Ras protein (membrane bound) [16]. Even inhibition of the post-translational modifications by Ras might lead to suppression of tumor cell proliferation [16].

β -catenin mutations have been documented in a variety of human malignancies, for instance, 10–14 % in colon carcinomas, 33 % in ovarian carcinomas, 4 % in medullo blastomas and 6 % in prostate carcinomas. Upregulation of the β -catenin gene devoid of exon 3 has been known to transform cellular morphology. Nuclear β -catenin accrual was frequently found in squamous differentiation stage of endometrial cancer. β -catenin abnormalities could play key roles in

tumor cell differentiation [17]. MNNG promotes dissociation between E-cadherin and β -catenin, and as a result, β -catenin expression is upregulated. MNNG activates Ras/MAPK pathway is an oncogenic dependent way [18]. Our present results suggest that GOH could influence the potential engrossment of the β -catenin pathway. GOH prevents EC by forming intermediates and phosphorylation of β -catenin and other serine/threonine residues. In a vicious cycle, the activated MAPK may be attributed to the increased synthesis of reactive oxygen species and production proinflammatory cytokines. GOH lowered MAPK level, corroborating the previous findings [19]. Therefore, the GOH induced suppression of the elevated β -catenin expression/content and MAPK levels might leads to decreased ROS generation.

Absence of E-cadherin expression was coupled with differentiation and spreading in endometrial tumors in various organs. Cadherins play crucial roles in the maintenance of cell–cell junctions by a calcium-mediated cellular mechanism [20]. In several malignancies, diminished E-cadherin expression is associated with flawed cell–cell adhesiveness, thus promoting invasion and metastasis. Subsequent to the loss of E-cadherin, β -catenin localizes into the nucleus and induces MAPK gene. Our results corroborate the finding that MNNG reduces the expression of E-cadherin by activating β -catenin and MAPK pathway [21].

The PI3K pathway is initiated in more than 80 % of EC cells and mutations affecting pathway holding great potential for different treatment strategies for its disease [22]. In EC, PI3K pathway is assessed by AKT phosphorylation which is associated with the presence of PTEN mutation or K-ras mutation. Burke et al. [23] proposed that GOH could inhibit cell proliferation by ornithine decarboxylase activity. The present study adds evidence indicating that GOH could act as a potential anticancer agent by inhibiting cell proliferation process.

MNNG induced activation of TGF- β which initiated PI3K activation and increased AKT phosphorylation/

Table 2 Western blotting analysis of PCNA, PTEN, Progesterone and E-cadherin expression in endometrial carcinoma

RT-PCR markers	Control	MNNG	GOH	MNNG + GOH (Pre-treatment)	MNNG + GOH (Co-administration)
PCNA	1.1 ± 0.07	2.5 ± 0.12 [#]	1.3 ± 0.07	1.62 ± 0.09**	1.9 ± 0.11**
PTEN	1.03 ± 0.06	1.51 ± 0.09 [#]	1.8 ± 0.1	1.74 ± 0.1**	1.64 ± 0.08**
Progesterone	0.97 ± 0.04	1.28 ± 0.07 [#]	1.77 ± 0.08	1.61 ± 0.09**	1.47 ± 0.06**
E-cadherin	1.13 ± 0.08	1.36 ± 0.08 [#]	1.92 ± 0.11	1.78 ± 0.08**	1.53 ± 0.1**

The overexpression of PCNA was detected in MNNG induced rats. Pre and co-administration of GOH shows reversed expression. PTEN, Progesterone and E-cadherin expression was downregulated in MNNG treated groups. During GOH treatment (pre and co-administration) upregulation of PTEN, Progesterone and E-cadherin expression was noticed. The mean ± SD are indicated (SD as error bars). [#] (MNNG) significantly ($p < 0.05$) different from normal groups, ** (MNNG + GOH) significantly different from MNNG group

activation; TGF- β elevated the invasiveness of EC through the activation of PI3K [24]. GOH inhibits Ras/MAPK pathway and MEK/ERK phosphorylation lead to down regulation of TGF- β pathways. The present study adds evidences for the modulation of these pathways and their downstream events. Uncontrolled proliferation and cell cycle dysregulation are widespread in almost all types of cancer. Progesterone is mainly used for primary treatment of endometrial cancer because it inhibits endometrial carcinogenesis by inducing apoptosis and down regulates wnt signaling pathway and TGF- β [25]. Loss of PR is related with increased expression of PCNA which is an effective marker for EC. The downregulation of PCNA expression by GOH could inhibit proliferation, reduce redox homeostasis and dysregulate oncogene expression. In normal endometrium, PR is known to restrain the proliferative actions of oestrogen through ER gene expression [26]. The loss of PTEN expression in EC is owing to gene mutations, gene methylations and various causes including abnormal PTEN post-transcriptional regulation in addition to aberrant mechanism of PTEN protein stability and degradation [27]. GOH has ability to decrease DNA synthesis, which influences the progression of cells through the S phase of cell cycle in colon cancer. GOH is known to down regulate the PTEN, PCNA, MAPK signal transduction pathway in corticomedullary tubular cells thereby inhibiting proliferation and HMGC-A reductase [28]. Our results added evidences on the effect of GOH on endometrial carcinoma.

Conclusion

The present results suggest that, GOH could act as a potent antiproliferative agent on MNNG induced endometrial cancer. GOH has no cytotoxic effect by protecting cells and tissues from the cytotoxic effect of carcinogens. Inhibition of PCNA expression by GOH is one of the several targets in its antiproliferative actions. GOH also up regulates PR which is mainly involved in female reproductive

functions. These results provide powerful evidence that GOH could influence modulation of K-ras, MAPK, PI3K, Wnt/ β -catenin and TGF- β pathways prevents endometrial carcinoma.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest.

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