Carvacrol Alters Soluble Factors in HCT-116 and HT-29 Cell Lines

Abstract

Aim: Natural products are a popular insight for researchers to investigate promising anti-cancer agents since some of these substances have lesser adverse effects restricting the treatment than traditional chemotherapeutic agents. A well-known monoterpene Carvacrol, widely consumed in Mediterranean cuisine and related to lower risks of cancer, has efficient anti-cancer effects. However, the mechanism of action is yet to be discovered.

Methods: The investigation aims to illuminate a new perceptive in the role of this substance on colorectal cancer treatment, by the means of differences in a well-defined range of soluble factors. Carvacrol effect on both HT-29 and HCT-116 cell lines were evaluated on proliferation and the IC$_{50}$ values calculated by the RTCA xCELLigence device. Then MAGPIX assay was performed to obtain the changes in soluble factors of the cell lines.

Results: The Multiplexing assay suggests some of these factors altered in favor of surviving and proliferation in aggressive cell line HCT-116 whereas they altered against these characters in HT-29, correlated with the increased IC$_{50}$ concentration of HCT-116 in carvacrol treatment.

Conclusion: The current study indicates that differences in the levels of these soluble factors could modulate the anti-cancer effect related to carvacrol.

Key Words: Carvacrol, HCT-116, HT-29, Prolactin, sVEGFR-2, Leptin, GM-CSF.
1. Introduction

Colon cancer has become a major public health issue with 1,849,518 new cases in 2018. 10.2% of all cancer deaths in the same year were caused by this malign disease which brings it to the second rank in cancer-related deaths. (1) Several factors (i.e. age, familial cancer syndromes, inflammatory bowel disease, dietary factors, hereditary polyposis conditions) affect the development of this malignancy. (2) Nowadays, the most accepted therapies are surgery, radiotherapy, and chemotherapy such as 5-fluorouracil and oxaliplatin. (3,4) Whereas all these procedures have their own difficulties, the adverse effects related to systemic chemotherapy are much restrictive due to their mortality. Since some of the natural products as chemotherapeutic agents indicate lower ill effects on patients, they are promising anti-cancer agents and possible sources of safer cancer treatments. Moreover, such phytochemicals are interested as also chemo-preventive agents because of their efficiency and low toxicity profiles. So the scientists focus on evaluating phytochemicals with multi-targeted potentials for effective chemoprevention. (5)

Terpenes (C10H16) illustrated enormous effectiveness as chemopreventive agents in the last years however the mechanism of action is not clear yet. (A subclass of this family, terpenoids, has been characterized as monoterpenes, diterpenes, oligoterpenes, and polyterpenes.) They involve roughly 25,000 chemical structures which are used in pharmaceutical industries. (6) Carvacrol is also a member of this family. The substance presents in various sources that are extensively tested with enormous biological activities.
(7) Mediterraneans regularly consume this product within oregano spice that presents a lesser risk of colorectal cancer compared to other local cuisines. (8) Nowadays, it's well known the carvacrol in this spice is the one reason that the substance has preventive properties. It basically protects mammalian cells against DNA strand breaks along with rosmarinic acid. (9, 10) Moreover, several studies point out that carvacrol illustrates further anti-cancer effects such as genotoxic, cytotoxic and proapoptotic activity to cancer cells, in a dose-dependent manner. Additionally, it has also an important preventive role in cell invasion by down-regulating of matrix metalloprotease 2 and 9 expressions. (11)

Tumor microenvironments involve various cell types and extracellular matrix substances in the niche of cancer such as soluble factors. The soluble factors which could be related to cell proliferation are Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Leptin, Prolactin and, soluble Vascular Endothelial Growth Factor Receptor-2 (sVEGFR2) evaluated. (12) Prolactin may involve in the genetic and molecular mechanisms for regulating the proliferation and growth of neoplastic and normal epithelial cells in the gastrointestinal tract. (13) Moreover, leptin is crucial for colorectal cancer (CRC) growth in obese patients. (14) On the other hand, sVEGFR-2 involves the extracellular domains of the receptor however lacks the tyrosine kinase domain. (15) GM-CSF has an important role in hematopoiesis and immune modulation. GM-CSF also stimulates the progression of immune-independent tumors by inducing tumor growth, metastasis, and promoting tumor microenvironments. Hence several studies suggest that this factor triggers some effects on tumor progression. (16)

Since these soluble factors manage the tumor microenvironment and so the behavior of cancer as mentioned previously, the present study investigates changes in these soluble factors when HT-29 and HCT-116 colorectal adenocarcinoma cell lines were treated with
carvacrol in a dose-dependent manner. We suggest the results could lead us one step further to discover the mechanism of action of carvacrol through the soluble factors.

2. Materials and Methods

2.1. Cell Culture

Human colorectal carcinoma, HCT116 (ATCC CCL-247) and HT-29 (ATCC HTB-38) cell lines were purchased from American Type Culture Collection (ATCC) (Rockville, CT, USA). They were cultured in McCoy’s-5a modified medium (BiochromGmbH, Berlin, Germany) supplemented with 1% penicillin/streptomycin (BiochromGmbH, Berlin, Germany) and 10% fetal bovine serum (FBS, Cegrogen Biotech GmbH, Stadtallendorf, Germany). Cells were incubated at 37 °C in a humidified 5% CO₂ incubator.

2.2. Cell Viability

The anti-proliferative effect of carvacrol (C7727, Sigma-Aldrich, St Louis, MO, USA) has been studied on HCT-116 and HT-29 colorectal adenocarcinoma cell lines. The cell lines were seeded in a triplicate in 96-well plate (10000 cells per well). Cell viability was tested after 48 hours of incubation with 25-200 µM carvacrol via xCELLigence Real-Time Cell Analysis Instrument (ACEA Biosciences, Inc (ACEA) San Diego, California, USA.)

2.3. Analysis of Soluble Factors via Multiplexing Assay

Colorectal adenocarcinoma cell lines, HCT-116 and HT-29 were treated with carvacrol at IC₅₀ values individually. Both carvacrol treated and non-treated control groups were seeded into wells and run in triplicate. The fluorescent signal was measured
by a CCD imager and the concentrations of the analytes were determined by MAGPIX xPONENT software. The results were normalized to total protein concentration estimated with Bradford assay.

2.4. Statistical Analysis

SPSS (Version 24.0; SPSS, Inc., Chicago, IL, USA) was used for all analyses. Willcoxon Signed Ranks non-parametric test was used to determine the significance between the application of different doses of carvacrol on different cell lines individually and its control group. The data in the figures are represented as the mean ± standard error of the mean. In the study, all tests were performed in triplicate. The level of statistical significance was set at P<0.05.

1. Results

The current investigation draws attention to the carvacrol effect in proliferation and also points out its mechanism of action through the alterations in the level of soluble factors in colorectal cancer cell lines with different phenotypes. The methodology indicates the IC50 values for carvacrol and follows through the MAGPIX multiplexing assay to illuminate the changes in soluble factors in response to IC50 values of carvacrol on HCT-116 and HT-29 colorectal cancer cell lines.

3.1. The Effect of Carvacrol on Cell Proliferation of Colorectal Cancer Cells

The \textit{in vitro} proliferation activity doses of carvacrol on HCT-116 and HT-29 cell lines were measured by using the xCELLigence Real-Time Cell Analysis Instrument. After 48 hours of incubation with carvacrol, IC50 values were found as 92 µM for HCT-116 and 42 µM for HT-29 cell lines. The results have been illustrated in Figure 1.
3.2. Carvacrol Effect on Soluble Factors in Colon Cancer Cell Lines with Different Phenotype

The soluble factors were analyzed to evaluate the cellular response leads to cytotoxicity in both cell lines and the difference between the response intensity to carvacrol. The measurements in samples were achieved by MAGPIX multiplexing assay. (Table 1) The normalized data illuminates that the secretion of several soluble factors related to growth and survival such as prolactin, leptin, and GM-CSF were increased in carvacrol treated HCT-116 while they were decreased in carvacrol treated HT-29 comparing to non-treated control groups. Prolactin, leptin and GM-CSF concentrations measured as 57.5, 9 and 82 pg/ml in HCT-116 and, 147, 17.74 and 104 pg/ml in HT-29 with no carvacrol concentration. These values raised in concentration to 117, 23, 105 pg/ml ($p=0.028$ for all concentrations), in response to 92 µM, the IC50 value of carvacrol, respectively. Whereas they decreased to 53.49, 14.49 and 96 pg/ml concentrations ($p=0.028$ for all concentrations) compared to the control concentrations in HT-29 treated with 42 µM of the phyto-compound. However proliferative and survival factor TGF-α decreased in both cell lines, correlated with earlier proliferation assay. This factor was estimated as 67 pg/ml for HCT-116 and 106.2 pg/ml for HT-29. Both values diminished where the concentration for HCT-116 treated with 92 µM substance was 24 pg/ml ($p=0.028$) and 26 pg/ml ($p=0.109$) for HT-29 treated with 42 µM of carvacrol.

2. Discussion

The present study focused on the effect of carvacrol in proliferation and also illuminate its mechanism of action through the change in the level of soluble factors in
colorectal cancer cell lines with different phenotypes. Both of these cell lines are treated with different doses of carvacrol which are 25 µM, 50 µM, 100 µM, 200 µM in xCELLigence Real-Time Cell Analysis instrument which measures the proliferation once per 15 minutes. The results signify that the proliferation levels of both cell lines decreased while the concentration level of carvacrol increased. A previous study points out that a carvacrol rich essential oil of origanumacutidens has also significant anti-proliferative effect against the HT-29 cell line. The most active concentration of this oil has been found as 100 µg/mL. (17) Moreover, similar to our results, Kai Fana et. al have studied the anti-proliferative effect of carvacrol on HCT-116 and they detected the IC₅₀ dose as 544.4 µM which is even higher concentration comparing to our result. (11) Even though carvacrol has a significant anti-proliferative effect on both of these cell lines in the dose-manner, our results indicate that it is more efficient against HT-29 since IC₅₀ value of HCT-116 doubles the other line.

The data of proliferation assay on the carvacrol effect point out that the phyto-compound has a significant role in both HCT-116 and HT-29 cell lines. However, the intensity of the cytotoxic responses is different since the more concentrated dose is required to achieve the in HCT-116. Seeing that the result, the cellular response was evaluated by the means of secreted soluble factors via multiplexing assay. (Table I) The normalized data illuminates that the secretion of several soluble factors such as prolactin, leptin, and GM-CSF was increased in carvacrol treated HCT-116 while they were decreased in carvacrol treated HT-29 comparing to non-treated control groups. Considering this mitogenic effect and survival character more abundant in HCT-116 whereas it reduced in HT-29 in response to the Phyto-compound, HCT-116 could be evaluated more resistant and aggressive in carvacrol treatment for that reason.
Nevertheless, a significant decrease in proliferative and survival protein TGF-α, in addition, the increase in anti-growth factors VEGFR 2 were correlated with the total cytotoxic effect in the final response.

The literature mostly confirms the results which we have obtained in the current study. The sVEGFR-2 fragments which are transfected to tumor cells secreted to the extracellular matrix and inhibit angiogenesis in vivo. (18) Another soluble factor increased in HCT-116, prolactin, actively involved in tumorigenesis in several cancers. The current research also points out that the prolactin level of HT-29 is dramatically lower compared to the control group. On the other hand, the level of this factor enormous enhanced in the HCT-116 cell line. Moreover, the scientists have investigated in improvement cures to control tumor growth via reducing the prolactin production. (19) The data introduced in the current study illuminates that levels of prolactin increased in HCT-116 as a result of favoring survival and proliferation, whereas it was decreased in HT-29. Therefore this growth-favored factor indicates that it is a possible target to enhance carvacrol treatment. In addition, the expression level of leptin was measured in lower levels in the HT-29 cell line comparing to its control group. However, HCT-116 has an elevated level of this soluble factor. Since, previous reports suggest that leptin is over-expressed in various types of cancer cells and plays a role in the development and progression of a variety of malignancies including colon cancer, our current study also reveals another reason why HCT-116 cell line was more resilient than HT-29 against this compound. (20,21) One more soluble factor GM-CSF was another growth factor decreased in HT-29 whereas increased in HCT-116. On account of the earlier studies indicating that neutralizing GM-CSF reduced the proliferation, angiogenesis, and colonic epithelial cells (CECs) in
neoplasia, our data point out that this factor is also an accessory after the fact along with leptin and prolactin. (22)

In conclusion, different colorectal cancer cell lines are responding in distinct ways to this phytochemical compound. On one hand, the anti-proliferative feature of carvacrol has been shown in both cell lines, on the other hand, the HCT-116 response more resistant to carvacrol treatment than HT-29 cell line. On the basis of present data, our results revealed that the alteration in levels of soluble factors in the HCT-116 cell line enhances the ability to proliferate and survive. Even though some of these factors slightly favored to tumor growth for HCT-116, carvacrol affects both cell lines as an anti-proliferative agent. Therefore this study also suggests some soluble factors could be drug targets to enhance the effectiveness of carvacrol.

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Figure 1.

The anti-proliferation effect of carvacrol in colorectal cancer. Dose-dependent cytotoxicity activity of carvacrol on HT-29 (A). The HCT-116 cell line was incubated with increasing concentrations of curcumin (B). After 48 hours of incubation at 37°C and 5% CO2 the cell viability has measured by xCELLigence Real-Time Cell Analysis instrument.

Table 1. Statistically significant concentration levels of soluble factors in carvacrol treated samples comparing to non-treated controls. All measurements were achieved by
MAGPIX multiplexing assay. Red indicates a decrease whereas green illustrates an increase in concentration compared to controls.