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EXPERIMENTAL STUDY

The detection of curcumins' antitumoral effects via argyrophilic nucleolar organizing region-associated protein synthesis in mice with ehrlich's ascitic carcinoma

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ABSTRACT

BACKGROUND: Curcumin is a polyphenol compound that has antioxidant, anticancer, anti-inflammatory, anti-hyperlipidemic and antimicrobial effects. Nucleolar-organizing regions are the sites of the gene on chromosomes. The present study was aimed to show the antitumoral effect of curcumin via AgNOR protein synthesis in Ehrlich's ascitic carcinoma (EAC) bearing mice.

METHODS: Twenty three mice with EAC were randomly divided into 3 groups as positive control (n = 7), group 2 (n = 8) and 3 (n = 8) treated intraperitoneally with curcumin (25 mg/kg) and (50 mg/kg), respectively. The animals were sacrificed on Day 16, the solid tumors were removed out. Then, total AgNOR area/nuclear area (TAA/NA) and the mean AgNOR number were estimated for each mice.

RESULT: Statistically significant differences were determined among the whole groups for TAA/NA ratio (p = 0.000), conversely mean AgNOR number (p = 0.361). When comparingthe two groups; while no difference was determined between the control and curcumin (25 mg/kg) groups (p = 0.061), the significant differences were detected between the control and curcumin (50 mg/kg) groups (p = 0.000) and between curcumin (25 mg/kg) and curcumin (50 mg/kg) groups (p = 0.000) for TAA/NA ratio. However, there was no significant difference for the mean AgNOR number in double comparison of the groups.

CONCLUSIONS: The current study showed that curcumin had a crucial function against cancer development. Also, both AgNOR values might be used as biomarkers for detection of the most reliable therapeutic dose selection of cancer treatment *(Tab. 3, Fig. 2, Ref. 27)*. Text in PDF *www.elis.sk.*

KEY WORDS: curcumin, NOR, AgNORs, rDNA, EAC.

Introduction

Despite modern advances in medical therapeutics worldwide, deaths from cancer have been increasing primarily due to lifestyle changes in the developing world. Recently, as cancer rate increases, the performed researches have specifically focused on the disease prevention. Many treatment options for cancer exist such as: radiation therapy, surgery, chemotherapy, hormonal therapy, immunotherapy, targeted therapy and palliative care. Epidemiological researches have also uncovered that diet and exercise may significantly impact the prevalence of specific types of cancer, renewing related with dietary phytochemical researches. Phytochemical agents constitute a heterogeneous set of bioactive compounds including alkaloids, polyphenols, carotenoids, and nitrogen compounds. These compounds are naturally found in vegetables, fruits, grains and other plant products and are generally responsible for several plant features such as: smell and color pigmentation (1). For a long time, the medicinal plants have been traditionally used to treat human disorders (2) and about 70 % of antitumoral drugs are natural products or their derivatives (3, 4). Curcumin, a bioactive compound derived from the rhizome Curcuma longa, has a chemotherapeutic and chemopreventive potential. This molecule has a polyphenolic features with an aromatic ring structure connected by two α, β-unsaturated carbonyl groups. Curcumin has different useful features such as: anticancer, anti-inflammatory, antioxidant, anti-hyperlipidemic and antimicrobial effects (5).

The Ehrlich ascites tumor cells are spontaneous murine mammary adenocarcinoma (6) and develop in almost all strains of mice as a rapidly growing carcinoma with very aggressive behavior (7). This tumor has similar features with human tumors and is sensitive to chemotherapy (8). Different studies indicated the beneficial use of Ehrlich's ascitic carcinoma (EAC) and solid tumor (EST) models as a valuable tool in exploring biological activities in cancer and evaluating the effect of several chemical compounds (7, 9, 10).

Nucleolar-organizing regions (NORs) are the ribosomal gene regions on chromosomes. These regions are composed of ribosomal DNA (rDNA) and proteins, some of which have argyrophilic features. These regions are transcribed into ribosomal RNA, which is converted into the preribosomes in the nucleolus and mature

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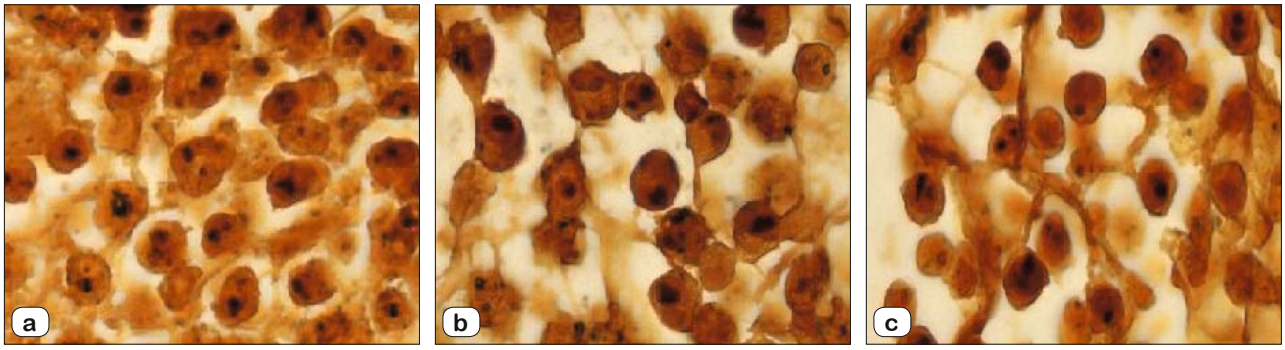

Fig. 1. A demonstrative examples of the AgNOR staining cells (a: positive control; b: Curcumin (25 mg/kg) group and c: Curcumin (50 mg/kg group).

ribosomes in the cytoplasm, respectively (11). Those regions can be stained with silver, when they are active. Due to their silver affinity, these proteins are called as argyrophilic NOR (AgNOR)-associated proteins and silver staining method is the most confident to indicate nucleoli in interphase nuclei (12) and to identify active NOR-bearing chromosomes at metaphase (13). There are various studies about the importance of the interphase quantity of AgNOR in tumor pathology, for the prognostic and diagnostic description of different cancer types (14, 15). To the best of our knowledge, no researches about the association between AgNOR proteins amount and the effects of curcumin treatment have been performed on EAC in literature. Thus we performed the current study to indicate any possible effects of curcumin treatment on the NOR protein synthesis and the selection of the most accurate dose for cancer treatment.

Methods

Experimental animals

All animal and experimental procedures were approved by the Experimental Animals Ethics Committee, Erciyes University, Turkey. About 6–8 week old Balb/c mice (average body weight of 25–30 g) were supplied from Laboratory Animal Unit of Experimental and Clinical Research Center, Erciyes University and kept under controlled conditioning (25±1 °C temperature, 55 % relative humidity and 12 h dark/ light cycles). Food and water were allowed ad libitum during the experimental period. Before commencement of the experiment, the mice were acclimatized to laboratory conditions for 7 days.

Tumor cells preparation and transplantation

The stocks animals with EAC were provided from Anatomy Department of Medical Faculty, Erciyes University. The tumor cells were maintained in our laboratory by serial intraperitoneal (ip.) passage in male Balb/c mice at 7–10 day interval. EAC cells were tested for viability using Trypan blue dye technique. Cell viability was often detected as 95 % or more. Tumor cell suspensions were prepared in Phosphate Buffered Saline (PBS).

Mice were inoculated subcutaneously at their back with $1x10^6$of EAC cells. Two hours after inoculation, twenty -three mice were randomly divided into three groups, and were treated as follows. The first group received vehicle injection (PBS) and served as EST positive control group (n = 7). Groups II (n = 8) and III (n = 8): were intraperitoneally exposed with curcumin (25 mg/kg and 50 mg/kg) during experimental process. All animals were sacrificed on Day 16, the tumors that developed at the site of injection were taken out and fixed in 10% formaldehyde and embedded in paraffin block for AgNOR staining.

AgNOR detection

The obtained tumor tissues were taken for routine histological procedures for AgNOR staining methods. The prepared slides were air-dried for 15 min at room temperature and fixed via fixative solution (3 : 1 ratio of methanol and acetic acid) for 5 min. AgNOR staining method was done according to literature with

Tab. 1. TAA/NA and the mean AgNOR number values of positive controls (n=7), curcumin (25 mg/kg) (n=8) and curcumin (50 mg/kg) (n=8) groups.

Groups	TAA/NA	Mean AgNOR number
Positive Control-1	0.198±0.068	2.020±1.116
Positive Control-2	0.179±0.115	2.040±1.160
Positive Control-3	0.178±0.063	3.039±1.483
Positive Control-4	0.146±0.043	2.122±1.013
Positive Control-5	0.129±0.041	1.520±0.647
Positive Control-6	0.143±0.175	2.000±0.728
Positive Control-7	0.153±0.062	2.280±0.904
Curcumin (25 mg/kg)-1	0.165±0.066	2.200±1.125
Curcumin (25 mg/kg)-2	0.170±0.072	2.060±1.058
Curcumin (25 mg/kg)-3	0.198±0.071	2.255±1.181
Curcumin (25 mg/kg)-4	0.142±0.058	1.878±0.807
Curcumin (25 mg/kg)-5	0.121±0.039	1.860±0.756
Curcumin (25 mg/kg)-6	0.136±0.046	1.880±0.773
Curcumin (25 mg/kg)-7	0.132±0.043	2.320±0.844
Curcumin (25 mg/kg)-8	0.130±0.039	2.360±0.827
Curcumin (50 mg/kg)-1	0.107±0.049	2.58±1.071
Curcumin (50 mg/kg)-2	0.115±0.038	2.62±1.123
Curcumin (50 mg/kg)-3	0.163±0.196	1.902±1.063
Curcumin (50 mg/kg)-4	0.179±0.150	2.000±1.155
Curcumin (50 mg/kg)-5	0.123±0.043	2.160±1.299
Curcumin (50 mg/kg)-6	0.136±0.058	2.080±1.226
Curcumin (50 mg/kg)-7	0.137±0.050	1.720±0.882
Curcumin (50 mg/kg)-8	0.147±0.080	1.820±1.189

TAA/NA – Total AgNOR area/Nuclear area

Tab. 2. Comparison of three groups for the mean AgNOR number and TAA/NAratio.

	TAA/NA	AgNOR Number	p	x^2
Positive Control	0.1609±0.0944	2.1486±1.11807	0.000*	36.669*
Curcumin (25µg/kg)	0.1555±0.06474	2.0233±0.97260		
Curcumin (50µg/kg)	0.1373±0.11068	2.2233±1.18242	0.361&	2.039&

*– for TAA/NA, & – for Mean AgNOR number, TAA/NA – total AgNOR area/nuclear area

Tab. 3. Double comparison of all groups for mean AgNOR number and TAA/NAratio.

Groups	For TAA/NA		For Mean AgNOR number	
	p	Z	p	Z
Positive Control -Curcumin (25 µg/kg)	0.061	–1.876	0.311	–1.012
Positive Control -Curcumin (50 µg/kg)	0.000	–5.806	0.751	–0.317
Curcumin (25 µg/kg)-Curcumin (50 µg/kg)	0.000	–4.221	0.176	–1.352

TAA/NA – total AgNOR area/nuclear area

a slight modification for all slides (16, 17). The AgNOR staining cells were investigated using a light microscope (Eclipse 80i, Nikon) and photographed with a digital camera (Digital Sight DS-fi1, Nikon). The images of the cells were transferred to image processing software (ImageJ version 1.47t, National Institutes of Health, Bethesda, Maryland, USA). Fifty nuclei were evaluated and, both total AgNOR area per nuclear area (TAA/NA) and the mean AgNOR number were calculated using the ''freehand selections'' tool for each nucleus. AgNOR staining cells were demonstrated in Figure 1 (a: control; b: Curcumin (25 mg/kg) group and c: Curcumin (50 mg/kg group).

Statistical analysis

Statistical analysis was carried out via the Statistical Package for Social Sciences (SPSS, Inc., Chicago, Illinois, USA) for Windows 22.0. The comparison of the groups (Positive Control, Curcumin (25 mg/kg) and Curcumin (50 mg/kg)) were performed using the Mann–Whitney U and Kruskall–Wallis tests and values (mean and standard deviation (SD)) were calculated via descriptive statistical methods. Results were given as the mean ± SD, and $p < 0.05$ was accepted as statistically significant.

Results

The TAA/NA ratio and the mean AgNOR number were detected in Curcumin (25 and 50 mg/kg) groups and positive control (Tab. 1). Statistically significant differences were detected among the three groups for TAA/NA ratio ($x^2 = 36.669$, $p = 0.000$) and the mean AgNOR number ($x^2 = 2.039$, $p = 0.361$) (Tab. 2, Fig. 2). When we performed to double comparison of the groups; while there was not a significant difference between positive control and Curcumin (25 mg/kg) groups ($Z = –1.876$, $p = 0.061$), the differences between positive control and Curcumin (50 mg/kg) groups ($Z = –5.806$, $p = 0.000$) and between Curcumin (25 mg/kg) and

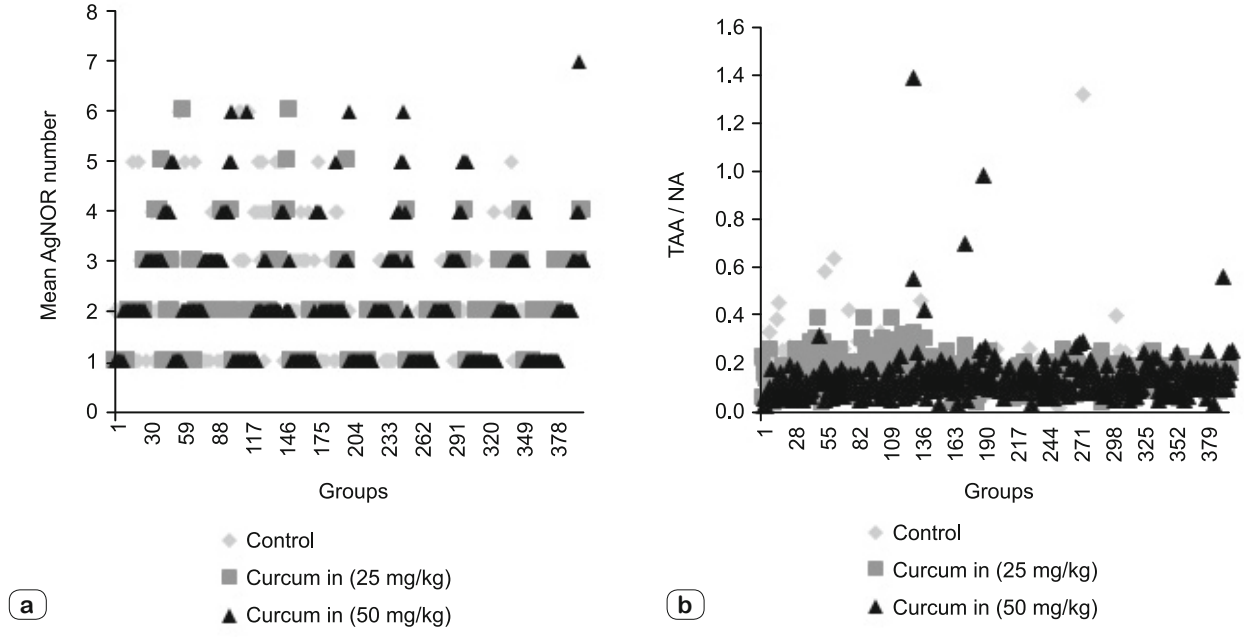

Fig. 2. Comparison of three groups for Mean AgNOR numbers (a) and TAA/NA values (b).

Curcumin (50 mg/kg) groups (Z = –4.221, p = 0.000) were significant for TAA/NA ratio. When we took into consideration the mean AgNOR number, there were not statistically significant differences between positive control and Curcumin (25 mg/kg) groups (Z = –1.012, p = 0.311), between positive control and Curcumin (50 mg/kg) groups (Z = –0.317, p = 0.751) and between Curcumin (25 mg/kg) and Curcumin (50 mg/kg) groups (Z = –1.352, p = 0.176) were significant for the mean AgNOR number (Tab. 3, Fig. 2).

Discussion

Cancer is one of the deadliest health problems worldwide. Therefore, alternative treatments such as: phytothrerapies have been used for cancer. Current chemotherapeutic agents' exhibit high toxicities and side effects: phytochemicals derived from plants offer safety and no side effects (18). Phytochemicals are naturally occurring substances found in plants. Several pre-clinical studies showed that natural phenolic compounds have anti-cancer activity. The phenolic components obtained from plants' products have played an important role in cancer prevention. Epidemiologic studies showed that the diets including high amounts of polyphenolic compounds are related with reduced cancer rates. Curcumin is used as a natural chemotherapeutic agent and it is a polyphenol extracted from the food spice turmeric (*Curcuma longa* Linn.) (19).

Curcumin has been widely researched for many pharmacological features. Also it had been studied in many tumors like colorectal cancers, prostate and rhabdomyosarcoma. Though curcumin is known to be nontoxic even at the dose of 3000 mg/kg body weight in rats, its bio-availability and stability inside the cell is very poor (20). Numerous scientific reports have shown that curcumin has anti-tumoral activity and targets different oncogenic pathways (19).

Curcumin inhibits cancer growth and progress, targeting different stages in the pathway to malignancy. It has activities such as blocking factor, inhibiting the initiation stage of cancer by suppressing agents and preventing carcinogen activation and malignant cell proliferation. Various animal researches have indicated that curcumin had a dose-dependent chemo-preventive effect in digestive systems such as oral, esophageal, stomach, duodenal and colon carcinogenesis. Curcumin did not only decreasethe count of tumors in each mouse and the percentage of mice with tumors, but also reduced tumor size. Also it was detected that there wa a marked preventive effect of curcumin on diethylstilbestrol (DES)-dependent promotion in radiation-initiated mammary tumorigenesis in rats (21). The nuclear factor kappa B (NF-kappaB) signaling pathway plays a crucial major role in not only cancer initiation but also cancer progression and promotion. The NF-kappaB protein interacts with DNA and cause transcription of genes that role in tumorigenesis, such as antiapoptotic, inflammatory, and cell proliferation and angiogenesis. NF-kappaB activation occurs mainly with I-kappaB kinase (IKK)–mediated phosphorylation of inhibitory molecules. Additionally, curcumin inhibits the NF-kappaB signaling and IKK activation, thereby suppressing proliferation of tumor cells. Curcumin also suppress different cell survival, cell proliferative genes and induced apoptosis. Thus, curcumin has chemo-preventive efficacy in almost all stages of tumorogenesis and nontoxic feature. Also, it was found that curcumin suppress NF-kappaB activation providing a beneficial effect by killing and preventing tumor growth in addition to inhibiting metastatic progression (22).

Thus, curcumin is a crucial natural product for developing a novel therapeutic strategy in human cancers. Therefore, metabolites such as curcumin obtained from natural plants are important sources for chemical synthesis and structural modification of new drugs and developing of new strategy for cancer treatments.

NORs have roles as functional subunits of the nucleolus and are related with a high number of regulatory proteins in interphase (23). These amounts of proteins also indicate the cellular metabolic activities. We carried various studies in benign and malign lesions (23–27). In those studies, we aimed to use the mean AgNOR number and TAA/NA ratio as a new approach in routine cytopathology for detection of the proliferation activity of cells in benign and malignant lesions. In this study, we purposed to show whether curcumin has an antitumor effects and whether AgNOR proteins amounts might be used for selection of the most reliable dose and detection of the new metabolites, which have a potential for the cancer treatments. To the best of our knowledge, this is the first research about the detection of AgNOR amounts in EST, which exposed the various curcumin concentrations. In the present study, when we compared the three group (Control, Curcumin (25 mg/kg) and Curcumin (50mg/kg)), although no significant differences were determined among the groups for the mean AgNOR number, there were statistically significant differences for TAA/NA ratio. In double comparison of the groups, while there were no statistically significant differences between the control and curcumin (25 mg/kg) groups; there was a statistically significant difference between the curcumin (50 mg/kg) and both the positive control and curcumin (25 mg/kg) groups for TAA/NA ratio. Thus, it might be said that the 50 mg/kg dose of curcumin is more reliable than curcumin (25 mg/kg) for the cancer treatments. Keeping in mind these findings, there were not statistically differences among the groups for the mean AgNOR number. The evaluation of AgNOR dots using a light microscope is subjective and poorly reproducible. Also, single AgNOR dots can be clustered together or overlapped. Additionally, the size of each silver-stained dot that is different does not take into the consideration when counting AgNOR alone. In cancer cells, in addition to gene expression, its' products, also cell morphology, both the number of biomolecules and the size of cells and their nuclei were changed, too. So, more reliable information about the proliferative and metabolic activity of the cells could be detected using the calculation of NOR area and nucleus area values. Description of new biomarkers for discrimination of benign and malignant lesions is important. Also, selection of the most reliable therapeutic strategy for cancer treatment is crucial for the management of treatment strategy to increase the success rate of therapy.

Our study indicated that the synthesis capacity of AgNOR proteins amount decreased depending on the exposed curcumin concentration. So, it might be said that curcumin has a significant role against tumor formation and suppress or trigger the synthesis of various proteins that have crucial function in the signaling transduction pathways and gene expression regulation in tumor cells.

Conclusion

Additional research including various metabolic, which have therapeutic features, should be done in various types of cancer to get information about the current topic. Hereby, the most reliable therapeutic treatment may be performed in the managements of the cancer. We detected that curcumin is an important molecule for prevention of cancer development. Also, present research showed that the estimation of TAA/NA ratio might be used as a biomarker to obtain information about the success rate of the performed therapeutic strategy and selection of the most reliable dose for cancer treatment.

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