EXPERIMENTAL STUDY

May argyrophilic nucleolar organizing region-associated protein synthesis be used for selecting the most reliable dose of drugs such as rhamnetin in cancer treatments?

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ABSTRACT

BACKGROUND: Rhamnetin is a flavonoid that has antioxidant, anti-inflammatory and anti-cancer effects. Nucleolar-organizing regions are the ribosomal genes region. We aimed to identify whether rhamnetin has an effect on cell proliferation and whether AgNOR proteins may be used for the detection of therapeutic benefits of the drugs and new metabolites, which have the potential of being used for cancer treatments.

METHODS: Twenty-four mice with Ehrlich’s ascites carcinoma (EAC) were randomly assigned to three main groups as positive control, and groups 2 and 3 treated intraperitoneally with rhamnetin (100 μg/kg and 200 μg/kg, respectively). All the animals were sacrificed on day16, 24 h after the last dose; the tumors, which developed at the site of injection were removed. Then, mean AgNOR number and total AgNOR area/nuclear area (TAA/NA) were detected for each mouse.

RESULTS: Significant differences were detected among all groups for mean AgNOR number (p = 0.000) and TAA/NA ratio (p = 0.000). While the difference between positive control and Rhamnetin (100 μg/kg) group was not significant (p = 0.387), there are significant differences between positive control and Rhamnetin (200 μg/kg) group (p = 0.000) and between Rhamnetin (100 μg/kg) and Rhamnetin (200 μg/kg) groups (p = 0.000) for TAA/NA ratio.

CONCLUSION: Rhamnetin has an important role in preventing cancer formation. Our study showed that mean AgNOR numbers and TAA/NA values may be used also as biomarkers for evaluating the success rate of the performed therapeutic strategy and accurate dose selection for the management of the disease (Tab. 3, Fig. 3, Ref. 45). Text in PDF www.elis.sk.

KEY WORDS: NOR, AgNORs, rhamnetin, cancer treatments, rDNA.

Introduction

Cancer is a major health problem throughout the world and the leading major cause of human mortality exceeded only by cardiovascular disease (1, 2). It is known that cancer treatment is usually based on surgical removal, radiotherapy, immunotherapy, hormonal therapy and chemotherapy, with the purpose of increasing the patients’ survival (3). Most of the currently available anticancer drugs fail to differentiate between normal and neoplastic cells or to overcome primary or secondary resistance mechanisms involved in cancer cells (4, 5). In this regard, there is an urgent need for novel pharmaceutical agents with tumor selectivity and specificity, but with limited side effects. The prevention of cancer through the ingestion of vegetables and fruits has been suggested in human epidemiologic studies, and the focus of drug development has been shifted to natural chemotherapeutic agents found in plants (6–8). Plants have been used as medicine for a long time and about 70 % of anticancer drugs are produced from natural products or their derivatives (9, 10). Rhamnetin, a flavonoid, is a bioactive polyphenolic compound, which is generally found in vegetables and fruits (11), and has a flavon nucleus consisting of two benzene rings combined by an oxygen-containing pyran ring. Rhamnetin is known to function as an antioxidant (12, 13) and alkylperoxyl radical-scavenging, (14) anti-inflammatory (15) xanthine oxidase inhibitory (16) and antiviral agent (17).

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The nucleolus is an important structure within the cell nucleus. Nucleolus is the site where ribosomal RNA and ribosomal subunits are made from proteins. The size of nucleolus and its organization are directly associated with ribosome production and exhibit the functional compartmentation of the nucleolar machinery.

Nucleolar-organizing regions (NORs) are the ribosomal genes region on chromosomes and is composed of ribosomal DNA (rDNA) and proteins, while some of them are argyrophilic. These regions are transcribed into ribosomal RNA, which processes the preribosomes in the nucleolus to become part of mature ribosomes in the cytoplasm (23). The NORs can be stained with silver when they are active. Thus, those proteins are referred to as argyrophilic NOR-associated proteins (AgNORs), while the silver-staining method is the most reliable to show nucleoli during the interphase (24). Numerous studies have been carried out on the importance of the interphase quantity of AgNOR proteins in hair root cells of humans (25, 26), buccal epithelial and blood cells of Down syndrome infants and healthy persons (27–29), possible effects of carbonmonoxide (CO) exposure on the AgNOR protein synthesis of the cells of the heart, lung and femoral muscle (30–33) and ischemia/reperfusion injury (34). However, to our knowledge, there is no study about the relation between AgNOR proteins and the effects of rhamnetin exposure on EST in literature. Thus, we carried out the current study to show this association.

Methods

Experimental animals

All animal procedures and experimental protocols were approved by the Experimental Animals Ethics Committee, Erciyes University, Turkey (13/146 – 11/12/2013). Balb/c mice, about 6–8 weeks old with an average body weight of 25–30 g were procured from Laboratory Animal Unit of Experimental and Clinical Research Center, Erciyes University and housed under controlled conditioning (25 ± 1 °C constant temperature, 55 % relative humidity, 12 h dark/light cycles). Food and water were allowed ad libitum during the study period. The mice were acclimatized to laboratory conditions during 7 days before the commencement of experiment.

Tumor cells preparation and transplantation

EAC cells were obtained 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 for 7–10 days. EAC cells were tested for viability and contamination using trypan blue dye exclusion technique. Cell viability was usually found to be 95 % or more. Tumor cell suspensions were prepared in Phosphate Buffered Saline (PBS).

Mice were inoculated subcutaneously at their back with 0.1 ml of EAC working suspension containing 1x10^6 of EAC cells. The day of tumor implantation was designed as day 1. Two hours after inoculation, 24 mice were randomly assigned to 3 main groups by 8 mice each, and treated as follows. The first group received a vehicle injection (PBS) and served as EST-positive control group. The mice from groups II and III were treated intraperitoneally with Rhamnetin (100 μg/kg and 200 μg/kg, respectively) every day. All the animals were sacrificed on day 16, 24 h after the last dose. The tumors, which developed at the site of injection were removed and fixed in 10 % formaldehyde and embedded in paraffin blocks for AgNOR staining.

AgNOR detection

The animals’ tissues were dissected (approximately 1 x 1 x 1 cm^3 in size). After routine histological follow up, the slides including 5-μm thick sections were prepared and deparaffinized in xylene and then rehydrated in graded alcohol solutions before AgNOR staining. The slides were air-dried for 15 min at room temperature and fixed using fixative solution (3 : 1 ratio of methanol and acetic acid) for 5 min. AgNOR staining method was carried out according to the Benn and Perle protocol, while the Lindner protocol was used with a slight modification for all slides obtained from three groups (35,36). The cells staining with AgNOR were viewed via a light microscope (Eclipse 80i, Nikon) and photographed using a digital camera (Digital Sight DS-fi1, Nikon). The captured 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 for each slide. The mean AgNOR number was counted and total AgNOR area per nuclear area (TAA/NA) was calculated via “freehand selection”.
### Discussion

Cancer is an important health problem all over the world. Therefore, different treatment strategies have been used for its management. One of them is based on phytotherapy, in which natural chemotherapeutic agents from plants such as rhamnetin are used. Rhamnetin is extracted from *Hippophae rhamnoides* Linn. It is a flavonoid compound also containing a polyphenol structure. It functions as a specific inhibitor of Notch-1 pathway via enhancing miR-34a level. The inhibition of Notch-1 activation may lead to anticancer effects. Therefore, rhamnetin has a role in the modulation of cancer cell survival (37). Hui Jia et al., reported that rhamnetin (at non-cytotoxic concentration) has an effect on enhancing the efficacy of anti-tumor agents via miR-34a-mediated Notch-1 suppression in hepatocellular carcinoma cells (HCC). Due to therapeutic effects of rhamnetin in the treatment of HCC, this molecule could provide the basis for developing a specific sensitizer of anti-tumor drugs (38).

Notch-1 also mediates the EMT (epithelial–mesenchymal transition) process that is associated with multi-drug resistance.
(MDR) or metastasis of human cancers (37). Thus, the inhibition of Notch-1 activation is an important strategy in the treatment of human cancer. Rhamnetin treatment increased the activity of sorafenib and traditional chemotherapeutic agents and also inhibited the EMT process of HepG2 cells (39). Rhamnetin is thus an important natural product for developing a novel therapeutic strategy in human cancers. Therefore, metabolites such as rhamnetin obtained from natural plants are important sources for chemical synthesis and structural modification of new drugs and development of new strategy for cancer treatments.

During the interphase, NORs are associated with a great number of regulatory proteins and they have roles as functional subunits of the nucleolus (40). Alterations in AgNOR protein amounts also reflect the metabolic activities of the cells. We performed various numbers of studies on malign and benign lesions (41–45). In these studies, we evaluated mean AgNOR number and TAA/NA ratio
as a new approach that may contribute to routine cytopathology for determining the proliferation activity of cells in malignant and benign lesions. In the current study, we aimed to identify whether rhamnetin has an effect on cell proliferation and whether the detection of AgNOR protein amounts may be used to detect the therapeutic benefits of the drugs and new metabolites that have a potential to be used in cancer treatments. To our knowledge, this is the first study on the evaluation of AgNOR amounts in EST that are exposed to different concentrations of rhamnetin. In our current study, when we compared the three (positive control, rhamnetin (100 μg/kg) and rhamnetin (200 μg/kg)) groups, significant differences were detected between them for mean AgNOR number and TAA/NA ratio. While the difference between Rhamnetin (100 μg/kg) and positive control group was not significant, statistically significant differences were found between rhamnetin (200 μg/kg) and both positive control and rhamnetin (100 μg/kg) groups for TAA/NA ratio. Therefore, it may be said that in cancer treatment, rhamnetin at a dose increased up to 200 μg/kg is more effective than when administered at a dose of 100 μg/kg.

When we consider mean AgNOR number, statistically significant differences were detected between positive control and rhamnetin (100 μg/kg), positive control and rhamnetin (200 μg/kg), and rhamnetin (100 μg/kg) and rhamnetin (200 μg/kg) groups for mean AgNOR number. Counting AgNOR dots under the light microscope is subjective and poorly reproducible. Additionally, single AgNOR dots can be clustered together or overlapped and counting alone does not take into consideration the size of each silver-stained dot that varies in amount. In metabolically active and cancer cells, not only gene expression and its products but also cell morphology, and synthesis capacity, as well as both number of biomolecules and size of cells and their nuclei were altered. Thus, more accurate knowledge about the metabolic and proliferative activity of the cells could be obtained by using new approaches based on the calculation of NOR area and nucleus area values. Identification of new biomarkers for discriminating benign and malignant lesions and the detection of the success rate of the performed therapeutic strategy is important for enhancing the diagnostic accuracy and management of treatment strategy for increasing the success rate of therapy.

The current study showed that the expression capacity of rRNA gene, as detected via total TAA/NA and/or AgNOR number per total nuclear number, decreased depending on the exposed rhamnetin concentration. It may be said that rhamnetin has an important role in prevention of tumor formation and triggers or suppresses the synthesis of some other proteins that have important features and functions in signaling the transduction pathways and gene expression regulation in tumor cells.

Conclusion

To obtain more accurate knowledge about the current topic, additional studies including those on different metabolic pathways that have therapeutic features should be performed in various types of cancer. In this manner, good therapeutic approaches may be developed for making the management of diseases more accurate. Additionally, because this technique is simple, cheap, and serves as a valuable marker to evaluate the ribosomal gene activity in different metabolic durations of various cells, it has important advantages.

It was detected that rhamnetin has an important role against cancer formation. The current study indicated that the detection of both the AgNOR values may be used also as a biomarker for detecting the success rate of the performed therapeutic strategy and selection of a reliable dose for accurate management of the disease.

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