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Original Article

Exposure to cigarette smoke alters AgNOR number and HIF-1alpha expression in colorectal tubular adenocarcinoma in rats

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Abstract: The relationship between exposure to tobacco and colorectal cancer development emerges in the long-term, leading to hypoxia and changes in the number of nucleolar organizer regions. There is evidence that this exposure influences on tumor characteristics, culminating in malignancy and poorer prognosis. This article aims at evaluating the influence of cigarette smoke exposure in HIF-1alpha hypoxia marker expression and AgNOR count, as well as the relationship between these two markers and tubular adenocarcinoma differentiation level in an experimental colorectal cancer model. Rats were induced colorectal cancer through 1, 2-dimethylhydrazine and randomly allocated into two groups: exposed and control. Exposed group was then, directly exposed to burning cigarette smoke. Tubular adenocarcinoma obtained was subjected to AgNOR counting technique and immunoblotted for HIF-1alpha protein. Smoke exposed groups had lower AgNOR numbers ($P = 0.00017$) and HIF-1alpha highest score ($P = 0.00017$). The average relationship between AgNOR and HIF-1alpha is weak in both groups and the differentiation level is not influenced by the AgNOR count in both groups. Notwithstanding this weak relationship, well-differentiated tumors in the control group have shown HIF-1alpha higher scores. In the smoke exposed group, as HIF-1alpha score increases, the tumor differentiation grade decreases. Thus, HIF-1alpha and AgNOR have shown themselves as biomarker study targets for diagnosis, prognosis and treatment response in colorectal tubular adenocarcinomas, since they tended to be related to the degree of tumor malignancy.

Keywords: NOR, carcinogenesis, neoplasia, hypoxia, rat, smoking

Introduction

According to World Health Organization [1], smoking is the leading cause of preventable death in the world and it is estimated that one third of the adult population, more than one billion people, are smokers. Tobacco is responsible for one death every six seconds and one in every ten deaths in adults, totaling 5.4 million deaths each year worldwide [1].

Furthermore, smoking is a risk factor for developing gastrointestinal cancer, including the oral cavity, esophagus, stomach, ileum and colon [2-6]. Specifically, colorectal cancer is the fourth most common cause of death worldwide with 694,000 incidents a year [1].

The influence of tobacco in colorectal cancer is clearer in the long term, since smoking for at least 20 years is significantly related to the emergence of small polyps. When that exposure exceeds 20 years, it is associated with large polyp's appearance and over 35 years exposure contributes to colorectal carcinoma onset [6].

The habit of smoking is related to NOR (Nucleolar Organizer Region) increase in smokers' oral mucosa compared to nonsmokers [7]. NOR is formed around certain chromosomal areas where three genes responsible for rRNA synthesis lye in repetitions in tandem. (18S, 5.8S and 28S) lying in tandem multiple repetitions [8].

In mice with colorectal cancer induced by 1, 2-dimethylhydrazine (DMH), a greater number of AgNOR (Argyrophilic Nucleolar Organizer Region) was found in tumors when compared to normal mucosa [9]. In this regard, DMH experimental models are widely used and share many similarities with colorectal cancer in humans, including response to inductive and preventive agents [10].

AgNOR count can be related to cell proliferation and differentiation. Specifically, a larger number of AgNOR in cell nucleus is linked to a lower cell differentiation degree and poorer tumor prognosis [11, 12].

Smoking for 10 minutes reduces the oxygen tension in tissues for approximately one hour [13]. In addition, a significant increase in hypoxia-inducing factors in cells exposed to cigarette smoke components were pointed out [14]. Cells undergoing hypoxia tend to decrease their division rate [15-17] and even shutdown the cell cycle in some tumors [18]. Low oxygen tension also alters cellular homeostasis, leading to HIF-1alpha protein activation, which expression is highly regulated by oxygen concentration and, for this reason, has been widely used as a main hypoxia marker [19-21]. HIF-1alpha expression in solid tumors may contribute to malignancy and aggressive behavior [22].

Given that exposure to cigarette smoke may induce changes in AgNOR and HIF-1alpha markers and these affect tumor characteristics such as malignancy and prognosis, this study aims to evaluating the influence of exposure to smoke on HIF-1alpha expression and the AgNOR count. It also intends to verify the relationship between these two markers, as well as the degree of colorectal tubular adenocarcinoma differentiation in an experimental model for colorectal cancer with DMH in order to assist other studies that use biomarkers as a diagnostic and prognostic tool.

Materials and methods

Ethical aspects

Animals used in this study were kept in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications Nº 8023), 2011 review, and with the Brazilian Law on Procedures for Scientific Use of Animals (#11794/2008). The experimental procedures were reviewed and approved by the internal Ethics Committee under protocol #003/2014.

Experimental design

The experiment used 24 male Wistar adult rats, kept in the Centre for Development of Experimental Models of Universidade Federal do Espírito Santo. Animals were housed in the number of 6 per cage under controlled temperature (21-24°C), humidity (45-55%) and lighting (12 h light, 12 h dark; lights on at 6:45 AM). Food and water were available *ad libitum* throughout the experiments.

All animals were induced colorectal cancer through 1, 2-dimethylhydrazine (DMH). DMH was dissolved in 0.9% NaCl containing 1.5% EDTA as a vehicle, adjusted to a final pH of 6.5 with 1 N NaOH solution and administered subcutaneously once a week for five weeks at a dose of 65 mg/kg body weight. This protocol was chosen based on previous data [23].

The 24 animals were then randomly divided into 2 groups, one directly exposed to smoke from burning cigarettes (exposed group) and another unexposed (control group). Exposure to cigarette smoke was started simultaneously with the induction of carcinogenesis and was carried out for 20 weeks in inhalation chambers equipped with trademark smoke puff.

After exposure phase, the animals were euthanized by anesthetic induction with a ketamine and xylazine association followed by the injection of supersaturated potassium chloride solution. They were then subjected to necropsy and their intestines were removed from the ceccum to the anus and opened with scissors at the mesenteric insertion for the removal of tumors.

The collected tissue was fixed in 10% buffered formalin solution and processed according to paraffin embedding routine. Following this stage, the blocks were cut in 3 μm thick histological sections, stained with hematoxylin and eosin and diagnosed according to Perše and Cerar [24].

For assessing the differentiation degree, AgNOR count and HIF1-alpha expression, the slides were analyzed in an optical microscope

Table 1. Number and percentage of intestinal mucosal lesions in an experimental model of colorectal cancer between exposed and not exposed to tobacco smoke groups according to their diagnoses

Lesion diagnosis	*Control Group*		*Exposed Group*	
	n	%	n	%
Mild dysplasia	-	-	1	3.03
Moderate dysplasia	2	8.0	3	9.09
High grade dysplasia	-	-	1	3.03
Carcinoma in situ	1	4.0	5	15.15
Tubular adenoma	2	8.0	-	-
Signett-ring cell adenocarcinoma	2	8.0	3	9.09
Tubular adenocarcinoma	17	68.0	20	60.61
Mucinous adenocarcinoma	1	4.0	-	-
Total	25	100	33	100

n: number of analyzed lesions, %: relative percentage to total number of characterized lesions.

by two pathologists, who received the same slides for analysis independently, without previous discussion of the findings. Their index of agreement was 99.1%, calculated according to the following formula: [AGREEMENT/(AGREED + DISAGREED)] × 100.

The degree of differentiation of each sample was determined by subjective analysis, considering the number of glands according to Fleming and cols. [25] to human colorectal tumors. That scale considers tumors formed by over 95% of glands as well differentiated; moderately differentiated tumors display between 50-95% of glands; and poorly differentiated tumors are composed mostly of solid parts with less than 50% of glandular formation. The lesions were then classified as benign or malignant neoplasm depending on their differentiation grade (well differentiated - 1, moderately differentiated - 2 and poorly differentiated - 3).

Staining and AgNOR count

From the selected sample, 3 µm thick histological sections were cut and then subjected to AgNOR staining technique as described by Ploton and cols. [26] with modifications.

The NOR count with silver staining (AgNOR) was performed with an optical microscope at 100× magnification under immersion. On each slide, AgNOR number was counted in 100 mucosa cells, in at least three fields of the neoplastic region, ignoring the edge and its adjacent zones.

Immunohistochemistry

The same tumors used for the AgNOR staining technique were used for immunohistochemistry. Paraffin-embedded blocks were sectioned and mounted on silanized slides. The 3-µm sections were deparaffinized in xylene and rehydrated through three baths in absolute alcohol. Slides were rinsed with deionized water and subjected to antigen retrieval with sodium citrate at high temperature for 15 minutes. Next, the slides were washed in 1× TRIS and endogenous peroxidases were blocked with 30% hydrogen peroxide in 1× TRIS for 20 minutes at 25°C. After three 5-minute washes in 1× TRIS, slides were incubated in nonspecific protein blocking solution (3% milk powder diluted in 1× TRIS) for 60 minutes at 25°C and subjected to more three 5-minute washes in 1× TRIS.

Control (no primary antibody) and experimental slides were incubated at room temperature, respectively, in 1× TRIS or antibody diluting solution with Anti-HIF-1alpha antibody (1:2000, [EP1215Y], AB51608, Abcam, Cambridge MA) for 60 minutes. After three 5-minute washes in 1× TRIS, slides were incubated with detection system for rat tissue (N-Histofine-Simple Stain Max PO Rat, 414191F, NICHIREI®) at 25°C for 30 minutes. After another three washes with onex TRIS, staining was visualized with peroxidase-sensitive Sigma fast 3, 3'-Diaminobenzidine (DAB, Sigma, St. Louis, MO). All slides were counterstained with Harris Hematoxylin for 1 minute, dehydrated in ethanol, cleared in xylene and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

The slides were analyzed in an optical microscope and sample scoring was performed by semi quantitative analysis, considering the number of stained cells and signal intensity. Considering the percentage of HIF-1alpha immune-positive cells, a score of 0 was given when all cells were negative; 1. when 1-25% of cells were positive, 2. when 25-50% of cells were positive and 3. when > 50% of cells were positive. Signal intensity was scored as negative (0), weak (1), moderate (2) and strong (3). Both scores were multiplied according to Soini

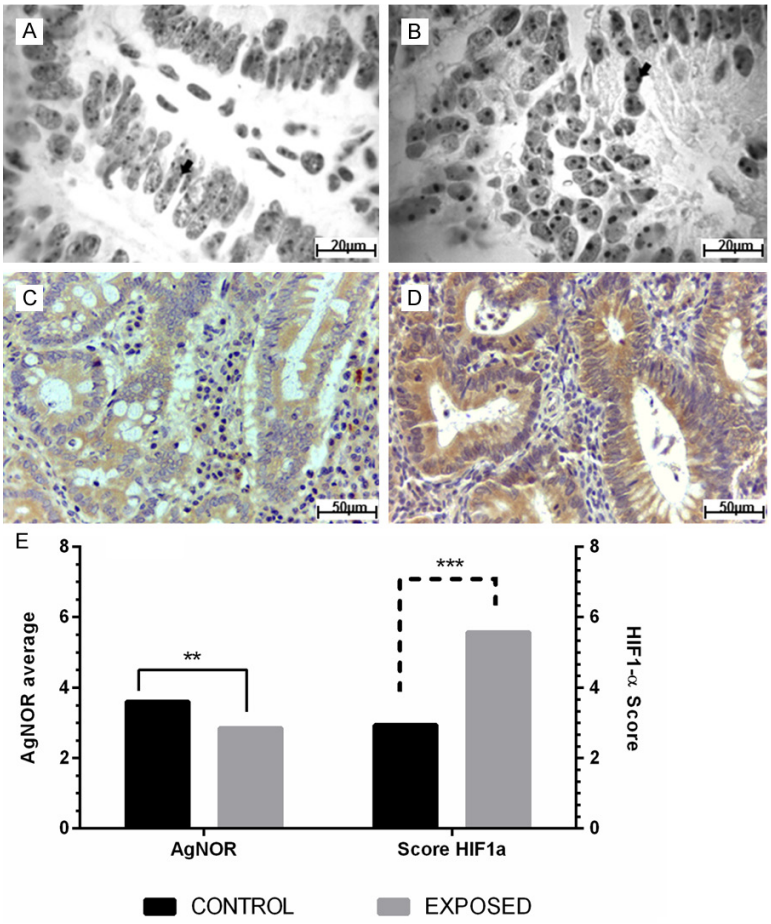

Figure 1. AgNOR average and HIF-1alpha score in colorectal adenocarcinoma tubular from experimental model for colorectal cancer exposed and no exposed to smoke cigarette. (A) Sample derived from control group and (B) sample belonging to exposed group showing the difference in the AgNOR number on cells (arrow). AgNOR staining. (C) Sample from control group classified with HIF-1alpha score 3. (D) Sample from exposed group classified with HIF-1alpha score 6. IHQ, DAB. (E) Significative difference between control and exposed groups by Student t test (**AgNOR average, P = 0.00017; ***HIF-1alpha score, P < 0.0001), α = 0.05.

and cols. [27] and Campos and cols. [28] and the resulting score was used in our analyses.

Statistical analysis

The data were submitted to analysis of variance, and the means of the treated samples and controls were compared using two-tailed Student's t-test (Program R©, 2015). Differences were considered significant for P < 0.05. This test was used for AgNOR and HIF-1alpha score. The Pearson Correlation test was used to evaluate the relationship between AgNOR and HIF-1alpha score, and then, its relationship with differentiation grade. Software GraphPad Prism 6 Demo was used for the drawing of graphics.

Results

All animals used in this experiment showed lesions in colorectal mucosa. In the exposed group, the minimum number of lesions was one and the maximum number of lesions found was nine, with an average of 2.75 injuries per animal. In the control group, 25 lesions were found with a mean of 2.08 per animal injuries. In this group, the minimum and maximum number of lesions was one and eight, respectively. **Table 1** shows the number of lesions and their diagnoses from which only Tubular Adenocarcinomas were selected, given their homogeneous characteristics, and due to the fact of being the most frequent injuries.

AgNOR number and HIF-1alpha expression in colorectal tubular adenocarcinoma

The overall AgNOR average per core on control group tubular adenocarcinoma was 3.6 ± 0.53 and on exposed group was 2.85 ± 0.51 demonstrating a significant difference (P = 0.00017). HIF-1alpha expression was higher in the exposed group when compared to the control group (P < 0.0001), with an average score of 2.94 ± 1.57 on control group and 5.57 ± 1.72 on exposed groups. All differences are illustrated in **Figure 1**.

Relationship between HIF-1alpha and AgNOR count and between both and the differentiation grade in colorectal tubular adenocarcinoma

In the control group, relationship between AgNOR average and HIF-1alpha score is weak negative (r = -0.2211; P = 0.3937), as well as in the exposed group (r = -0.1680; P = 0.4667), but not significant (**Figure 2A** and **2D**), as only 4.89% (control group) and 2.82% (exposed

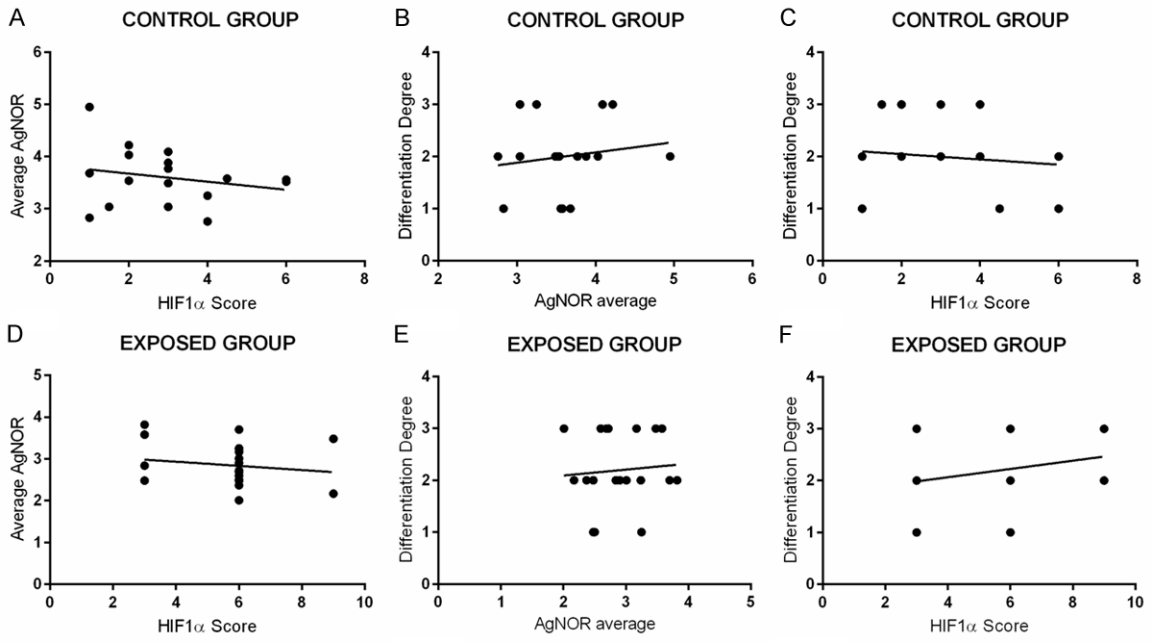

Figure 2. AgNOR and HIF-1alpha score relationship and its influence on colorectal tubular adenocarcinoma degree differentiation in smoke cigarette exposed group and no exposed group in colorectal cancer experimental model. A-C: Control group; D-F: Exposed group. Pearson Correlation, α = 0.05.

group) of change in AgNOR number can be explained by HIF-1alpha score.

The colorectal tubular adenocarcinoma differentiation grade in control group has shown little influence from AgNOR number (r = 0.1530, P = 0.5576), that is, only 2.34% of AgNOR is related to the worst degree of tumor differentiation. In the exposed group, relationship between AgNOR and tumor differentiation degree is much lower (r = 0.0861, P = 0.7106) and only 0.74% of AgNOR count can be related to the worst tumor differentiation degree in this group (**Figure 2B** and **2E**).

In the control group, tumors seem well differentiated according to HIF-1alpha score increases, but this ratio was only 1.27% (r = -0.1126, P = 0.6670). However, in the exposed group, 4.07% of HIF-1alpha score is related to differentiation grade of analyzed tumors, and, the higher the HIF-1alpha score, the lower the tumor differentiation grade (r = 0.2017, P = 0.3806) (**Figure 2C** and **2F**).

Discussion

The correlation between smoking and colorectal cancer has been recently described by Gross and Baranauskas [29] as being important for selecting diagnostic biomarkers and predicting the prognosis of smokers who have this type of tumor. Thus, our study evaluated HIF-1alpha expression and AgNOR count in an experimental model of colorectal cancer exposed to smoke from direct burning cigarette.

A previous study showed increased average number of AgNOR in colorectal cancer experimental model induced by DMH compared to the uninduced control group [9]. Our results showed that the average AgNOR count in colorectal adenocarcinoma induced by DMH group followed this increasing trend. However, when exposed to cigarette smoke, colorectal adenocarcinomas had lower average AgNOR count. We believe that the reduction in the mean number of AgNOR found in colorectal adenocarcinoma experimental model exposed to cigarette smoke in our study is related to the tolerogenic influence that exposure to cigarette smoke exerts on colorectal mucosa. That influence was mentioned in a review published by Cabral and Barbosa [30]. Thus, different carcinogens and co-carcinogens, time and type of exposure, like cigarette smoke, can affect the AgNOR analysis.

Cytological studies with AgNOR count in smokers and nonsmokers' oral mucosa showed an increase count in tobacco-exposed group [7,

31]. These results differ from those found in our study in terms of AgNOR count by cigarette smoke exposure, because our assessment was carried out in colorectal mucosa. However, increased AgNOR count in tobacco exposed group oral mucosa is probably related to type of cigarette smoke exposure, which is different from oral mucosa (direct exposure) to colorectal mucosa (indirect exposure).

It is known that hazard ratio between smoking and colon cancer is conditioned to the exposure period so that as higher the exposure, higher the malignancy degree [6]. The difference between the AgNOR averages in groups may be related to tumor malignancy. Yang and cols [32] showed differences in AgNOR average in benign and malignant human colon tumors, as well as Joyce and cols. [33] who reported difference between malignancy degrees of human's colorectal cancer according to its AgNOR count. In these works, the worst diagnoses are accompanied by increased AgNOR average. On the other hand, Rüschoff and cols. [34], Rayter and cols. [35] and Yamaguchi and cols [36] have found no difference between AgNOR average and the degree of malignancy in human colorectal tumors. Our results showed that there is a weak relationship between degree of colorectal tubular adenocarcinoma differentiation and AgNOR count in both control group and exposed group.

There is then a similarity between studies using AgNOR quantification as a characterization tool for malignancy and tumor prognosis of colorectal carcinomas. Therefore, a low AgNOR average in tubular adenocarcinomas in the exposed group may not be linked to a low malignancy compared to control group, but to other factors such as hypoxia.

During hypoxia, ribosomes production in nucleolus decreases, as a way of saving energy [37, 38]. Our results showed that in exposed tubular colorectal adenocarcinomas, AgNOR average was lower and HIF-1alpha expression was higher, suggesting hypoxia as a possible cause for this event. Interestingly, the results showed a weak relationship between these two markers.

As previously reported, smoking for 10 minutes lowers oxygen tension in tissues for approximately one hour [13], which could explain the higher scores of HIF-1alpha expression in the exposed group, demonstrating that tumors in rats exposed to cigarette smoke underwent hypoxia and that this information should be considered in diagnostic and prognostic issues of colorectal cancer.

Therefore, HIF-1alpha score expression relationship with colorectal tubular adenocarcinoma differentiation grade was evaluated. It was noticed that in the control group, whose HIF-1alpha expression was lower, there was a better tumor differentiation grade. However, poorly differentiated tumors were associated with higher scores of HIF-1alpha expression, as observed in the exposed group. Several pathological aspects of colorectal cancer as depth of the intestinal wall affected by the tumor, number of lymph nodes containing metastasis and metastasis to distant organs, may interfere with the survival of patients, and are usually related to a poorer prognosis [39, 40]. It has also been reported that high expression of HIF-1alpha in human colorectal cancer is associated with a poor prognosis [41] and as well associated with increased mortality, regardless of patient clinical characteristics and molecular variables [42]. In this sense, the expression of HIF-1alpha could provide important answers regarding the prognosis of smokers who develop colorectal cancer.

In conclusion, both AgNOR and HIF-1alpha have presented themselves as biomarkers, targets for study of diagnosis, prognosis and treatment response in colorectal tubular adenocarcinomas since they tended to be related to tumor malignancy grade.

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Disclosure of conflict of interest

None.

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