

The role of argyrophilic nucleolar organizing region-associated proteins in clinical exacerbation of chronic obstructive pulmonary disease

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Abstract

Objective: To investigate whether argyrophilic nucleolar organizing region-associated protein (AgNOR) parameters can be used as a biomarker that could potentially help with the management and clinical prognosis of chronic obstructive pulmonary disease (COPD) exacerbation.

Methods: This case–control study enrolled patients with COPD who were admitted to the Emergency Department and healthy sex- and age-matched control subjects. Peripheral blood samples were collected at hospital admission and the peripheral lymphocytes were silver-stained to investigate the quantity and distribution of AgNOR proteins. Fifty nuclei per patient were viewed and the total AgNOR area/total nuclear area (TAA/TNA) ratio and the mean AgNOR number for each patient were calculated.

Results: A total of 20 patients with COPD exacerbation and 17 healthy control subjects were recruited to the study. The TAA/TNA ratio and the mean AgNOR number were significantly higher in the patients with COPD exacerbation compared with the healthy control subjects. The mean AgNOR number showed a positive correlation with the pCO₂ levels on admission.

Conclusion: AgNOR protein levels were elevated during a COPD exacerbation compared with healthy control subjects and there was a positive correlation between pCO₂ levels and mean AgNOR number.

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Keywords

Chronic obstructive pulmonary disease, argyrophilic nucleolar organizing region-associated proteins, exacerbation, management, marker

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Introduction

Chronic obstructive pulmonary disease (COPD) is a cause of airflow obstruction that is commonly accompanied by progressing and deteriorating symptoms.¹ Patients with COPD commonly experience periodic exacerbations, which may lead to hospitalization or respiratory failure. An exacerbation of COPD is characterized by persistent clinical worsening with acute symptoms and is closely associated with high morbidity and mortality.²

A variety of causes and triggering conditions may lead to a COPD exacerbation and the subsequent clinical worsening may present with a range of severity. To diagnose clinical worsening and an exacerbation of COPD, clinicians mostly rely on pCO₂ retention levels and the patient's need for hospitalization.³⁻⁵ The fact that COPD is a complex disease with various pathogenic mechanisms that are affected by multiple noxious agents means that it is very important to understand its pathophysiology.^{6,7} The need for a clearer understanding of how genetic, epigenetic and environmental factors contribute to COPD disease progression has led to the investigation and use of new biomarkers.⁸⁻¹²

Previous studies have reported on predisposing factors for COPD, prognostic factors associated with COPD exacerbations and on biomarkers for the management of COPD exacerbations.⁸⁻¹² There is limited evidence in the literature of the use of an accessible marker that can be routinely applied in the clinical management of

COPD exacerbations. Therefore, there is a need for a novel approach to identifying a biomarker that could be used as a predictive indicator, which would help to identify those patients expected to have either a poor outcome or prognosis following a COPD exacerbation.

Some proteins and ribosomal DNA (rDNA) that are transcriptionally active compose of nucleolar organizing regions (NORs) on chromosomes and these active proteins are mostly argyrophilic.⁹ Transcribed rDNA that binds to silver, silver-stained NORs and argyrophilic NOR-associated proteins are collectively called argyrophilic nucleolar organizing region-associated proteins (AgNORs).⁹

As a technique, AgNOR staining is not specific for one particular protein. Various types of silver-binding ribosome-associated proteins may form AgNORs. Determination of the activities of these proteins has been used as a marker of proliferation and metabolic activity of cells; and studies have investigated AgNORs in different healthy and diseased organs.^{13,14}

Determination of the total AgNOR area/total nuclear area (TAA/TNA) ratio gives information about the proliferation rate of the nucleolar proteins and the cellular stress response to agents (e.g. toxicants, hypoxia).¹⁰ The simplicity and cost-effectiveness of the present AgNOR technique make this a valuable diagnostic method.^{14,15} In addition to the detection of the number, size and shape of AgNORs, the measurement of the total AgNOR area

and the total nuclear area used for the calculation of the TAA/TNA ratio is also important.¹⁴ Calculation of the TAA/TNA ratio reflects the protein synthesis capacity of the cells, which provides information about the metabolic and proliferative activity of each cell.^{14,15}

In light of the sensitivity of AgNORs to alterations in cell metabolism, this study investigated whether AgNOR parameters could be used as morphological biomarker that could potentially help with the management and clinical outcome of COPD exacerbation.

Patients and methods

Patient population

This case-control study enrolled consecutive patients with COPD that were admitted to the Emergency Department (ED), Düzce University Medical School, Düzce, Turkey with a clinical exacerbation between October 2016 and March 2017. Healthy sex- and age-matched individuals were recruited from the local population of Düzce, Turkey. Inclusion criteria were as follows: (i) adult patients previously diagnosed with COPD and admitted with dyspnoea; (ii) adult patients with COPD with clinical worsening. Exclusion criteria were as follows: (i) patients with a lack of data; (ii) patients with autoimmune or proliferative diseases; (iii) patients with septicaemia; (iv) patients that received exacerbation treatment before blood samples were taken in the ED; (v) patients that needed resuscitation.

This study received approval from the Ethics Committee of Düzce University Medical School, Düzce, Turkey before collection and use of the samples (no. 2016/101). All participants provided written informed consent.

Patient assessment in the ED

The patients were assessed immediately upon hospital admission and blood samples were taken within the first 10 min of arrival in the ED and before oxygen therapy. Whole blood (3 ml) was collected from the median cubital vein using a nonheparinized syringe and then immediately spread on a glass slide. Arterial blood pCO₂ levels on hospital admission were recorded. The clinical progress and the AgNOR parameters of the patients were recorded. Information related to hospitalization and discharge were recorded. Patients that were unresponsive to primary emergency treatment were hospitalized according to indications for hospital assessment or admission for exacerbations of COPD.¹⁶

Preparation of the slides and AgNOR protein staining

Blood samples obtained from the patients with COPD and the healthy individuals were dropped and spread on clean glass slides and dried at room temperature for 20 min. The slides were then fixed in methanol for 5 min. The lymphocyte nuclei were stained with silver immediately, which was a slight modification of the published protocols.^{17,18} The slides were incubated in the dark at 37°C for 15 min in a solution made by mixing one volume of 2% gelatin in 1% aqueous formic acid with two volumes of 50% silver nitrate. Then the slides were washed in distilled water.

Computer-assisted image analysis of AgNOR proteins

Silver stained blood samples were analysed and photographed using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) and a digital camera (Digital Sight DS-Fi1c; Nikon). Fifty nuclear AgNOR protein images per study participant were assessed and transferred to ImageJ version 1.47t

image processing software.¹⁹ With the help of a 'freehand selection' tool, measurements were obtained and the mean AgNOR number and TAA/TNA ratio for each nucleus were calculated.

Statistical analyses

All statistical analyses were performed using IBM SPSS® Statistics for Windows®, version 23.0 (IBM Corp., Armonk, NY, USA). All data are expressed as mean \pm SD unless otherwise stated. The mean nuclear AgNOR protein levels in the lymphocyte nucleoplasm and mean age between patients with COPD and healthy control subjects were compared using the Mann–Whitney *U*-test. The relationship between the clinical status of patients with COPD and both the mean AgNOR number and the TAA/TNA ratio were evaluated using polynomial regression tests. Receiver operating characteristic curve analysis was used to evaluate the sensitivity and specificity of AgNOR parameters for the detection of a clinical exacerbation of COPD. A *P*-value < 0.05 was considered statistically significant.

Results

This study enrolled 20 patients with COPD (mean \pm SD age, 72.20 \pm 9.92 years; range, 51–91 years) and 17 age- and sex-matched healthy control subjects (mean \pm SD age, 69.52 \pm 16.57 years; range, 38–88 years). Table 1 shows the demographic characteristics of the patients and the healthy control subjects. There was no significant difference in the age of the two groups.

The study analysed the silver-stained lymphocyte nucleoplasm from the patients and the healthy control subjects. The mean AgNOR number and the TAA/TNA ratio of the patients with COPD exacerbation were significantly higher compared with the healthy control subjects (*P* < 0.01 for both comparisons) (Table 2).

Table 1. Demographic data of the patients with chronic obstructive pulmonary disease (COPD) exacerbation and healthy control subjects.

Characteristic	Patients with COPD exacerbation <i>n</i> = 20	Healthy control subjects <i>n</i> = 17
Age, years	72.20 \pm 9.92	69.52 \pm 16.57
Age range, years	51–91	38–88
Sex, male/female	14/6	11/6

Data presented as mean \pm SD or *n* of patients.
No statistically significant between-group differences (*P* ≥ 0.05).

In the COPD exacerbation group, nine (45%; five females and four males) of the 20 patients experienced an acute clinical exacerbation that required hospitalization. All the patients were discharged after hospitalization and none of them died during hospitalization. Table 3 shows comparisons of pCO₂ levels and AgNOR parameters in patients with COPD exacerbation stratified according to their need for hospitalization. There were no significant differences in the mean AgNOR number and the TAA/TNA ratio between the patients with COPD exacerbation based on their need for hospitalization. The pCO₂ levels were significantly higher in the hospitalized patients with COPD exacerbation compared with patients with COPD exacerbation that were not hospitalized (*P* = 0.003).

Histological examination of the AgNOR staining showed marked differences in the amount and shapes of the silver-stained dots in the lymphocyte nucleoplasm between the patients with COPD exacerbation and the healthy control subjects (Figure 1). Most of the interphase nuclei of the lymphocytes from patients with COPD exacerbation were irregular shaped and contained large amounts of silver-stained dots.

Sensitivity and specificity analyses of AgNOR parameters demonstrated that the

Table 2. Argyrophilic nucleolar organizing region-associated protein (AgNOR) parameters for patients with chronic obstructive pulmonary disease (COPD) exacerbation and healthy control subjects.

AgNOR parameter	Patients with COPD exacerbation <i>n</i> = 20	Healthy control subjects <i>n</i> = 17	Z value	Statistical significance ^a
TAA/TNA ratio, %	0.470 ± 0.014	0.187 ± 0.044	-4.243	<i>P</i> < 0.01
AgNOR number	4.016 ± 0.574	1.662 ± 0.208	-4.243	<i>P</i> < 0.01

Data presented as mean ± SD.

^aGroups compared using Mann–Witney *U*-test.

TAA/TNA, total AgNOR area/total nuclear area.

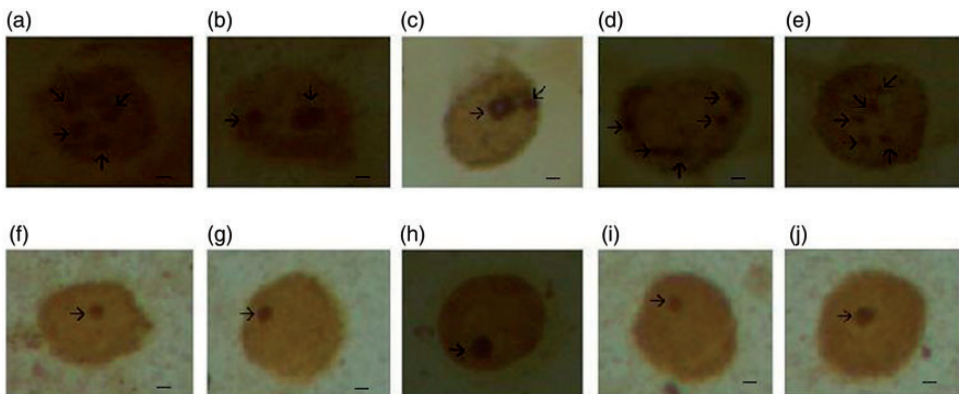
Table 3. Argyrophilic nucleolar organizing region-associated protein (AgNOR) parameters and pCO₂ values for patients with chronic obstructive pulmonary disease (COPD) exacerbation stratified according to their need for hospitalization.

	Hospitalized COPD patients <i>n</i> = 9	Non-hospitalized COPD patients <i>n</i> = 11	Z Value	Statistical significance ^a
pCO ₂ , (min–max)	69.8 ± 16.9 (40.7–97.2)	45.4 ± 7.4 (33.9–66.9)	-3.007	<i>P</i> = 0.003
TAA/TNA, %	0.194 ± 0.041	0.181 ± 0.047	-0.266	NS
AgNOR number	3.848 ± 0.521	4.153 ± 0.601	-1.178	NS

Data presented as mean ± SD and min–max.

^aGroups compared using Mann–Witney *U*-test.

pCO₂, partial pressure of carbon dioxide; TAA/TNA, total AgNOR area/total nuclear area; NS, no statistically significant between-group difference (*P* ≥ 0.05).

**Figure 1.** Representative photomicrographs showing peripheral lymphocytes with irregular shaped and varying size argyrophilic nucleolar organizing region-associated protein (AgNOR) dots inside the nuclei (arrows) of patients with chronic obstructive pulmonary disease (COPD) exacerbation (a, b, c, d, e). Representative photomicrographs showing peripheral lymphocytes from healthy control subjects with regular shaped cells and argyrophilic nucleolar protein (arrows) (f, g, h, i, j). ×1000, oil immersion. Scale bar 50 μm.

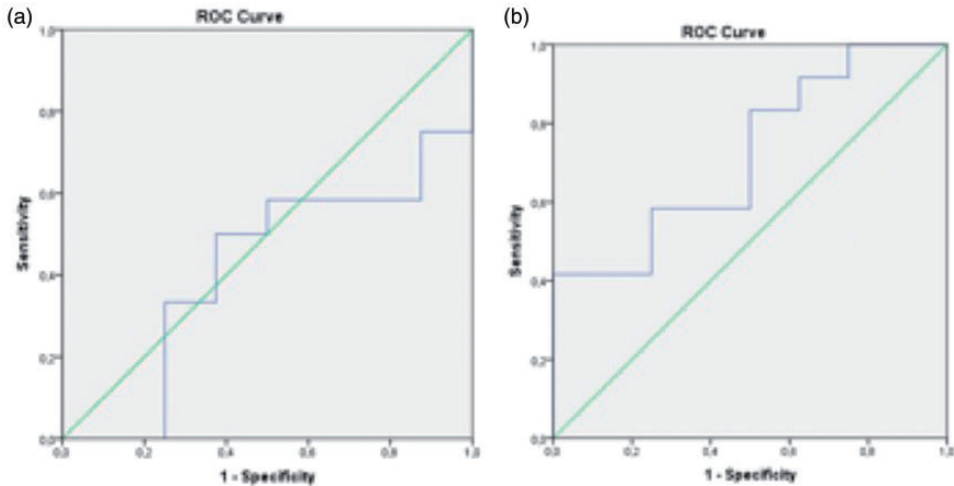


Figure 2. Receiver operating characteristic (ROC) curve analysis of argyrophilic nucleolar organizing region-associated protein (AgNOR) parameters in patients with chronic obstructive pulmonary disease exacerbation who were hospitalized ($n = 9$): (a) total AgNOR area/total nuclear area ratio; and (b) mean AgNOR number. The colour version of this figure is available at: <http://imr.sagepub.com>.

TAA/TNA ratio and mean AgNOR number provided 100% sensitivity and 100% specificity to distinguish patients with COPD exacerbation and healthy control subjects. The AgNOR parameters showed lower sensitivity and poorer specificity in predicting hospitalization of patients with COPD exacerbation (Figure 2).

The relationship between $p\text{CO}_2$ levels and the need for hospitalization showed a strong positive correlation ($r = 0.812$; $P < 0.001$). Also, there was a positive correlation between $p\text{CO}_2$ levels and mean AgNOR number ($r = 0.62$; $P = 0.03$).

Discussion

This current study demonstrated that the extent of AgNOR protein staining was a useful marker for distinguishing patients with COPD exacerbation from healthy control subjects and to a lesser degree for distinguishing patients with COPD exacerbation who had more severe disease that required hospitalization. To the best of our knowledge, this is the first published

study to assess the clinical associations between AgNOR protein status in the context of an acute COPD exacerbation. The TAA/TNA ratio was elevated in patients with clinically worsening COPD that were hospitalized compared with patients with COPD exacerbation who did not require hospitalization, although not significantly. The mean AgNOR number was lower in patients with clinically worsening COPD that were hospitalized compared with patients with COPD exacerbation who did not require hospitalization, although not significantly. The mean AgNOR number was positively correlated with $p\text{CO}_2$ levels, but could not be used to predict the decision to hospitalize the patient.

In order to identify any possible changes in cell biology in patients with COPD, this current study stained and evaluated 50 lymphocytes per patient with COPD, giving a total of 1000 analysed lymphocytes. Previous research has demonstrated that analysing 50–100 cells in more than 10 patients is sufficient to identify the

extent of AgNOR staining.^{13,14,20–22} Admittedly, a low number of patients make it more difficult to accurately analyse clinical correlations. However, the present study should be considered a preliminary investigation and studies with larger patient populations are required.

Exacerbations of COPD remain a challenge for clinicians in terms of both diagnosis and prognostic evaluation. None of the clinical or laboratory markers is optimal for monitoring the exacerbation of this disease.^{23,24} In addition, blood parameters are mostly abnormal at baseline and may not reflect acute events or dynamic alterations, so they are not used to monitor clinical improvement or recession in the disease progress.^{2,24}

This current study demonstrated that the nucleolar AgNOR protein TAA/TNA ratio in patients with clinical exacerbation that required hospitalization were, but not significantly, elevated compared with patients that did not require hospitalization. Both AgNOR parameters were significantly higher in the patients with COPD exacerbation compared with healthy control subjects, reflecting the severe systemic inflammatory reaction and cellular stress response at the time of the COPD exacerbation. Interestingly, the AgNOR number was elevated in non-hospitalized patients with COPD compared with healthy control subjects, theoretically indicating the impact of hypercapnia on baseline cell structure in these patients.

This current study aimed to not only investigate whether AgNOR protein staining is a potential marker for clinical COPD exacerbation, but also to determine if it can be used to predict the severity of the COPD exacerbation. This current study demonstrated that the AgNOR TAA/TNA ratio was elevated in patients with clinical exacerbation that required hospitalization compared with patients that did not require

hospitalization, but the difference was not significant.

This current study also demonstrated a positive correlation between pCO₂ levels and the mean AgNOR number. Based on this result, we speculate that the proliferation of AgNOR proteins reflects the systemic damage occurring as a result of hypercapnia during a COPD exacerbation. Exacerbation of this disease may cause irreversible damage to the cells by apoptosis and inflammation. Thus, AgNOR protein levels could potentially predict the long-term prognosis of a COPD exacerbation. Therefore, unlike some other acute markers, AgNOR reflects not only the severity of the exacerbation but may have potential implications for morbidity and mortality. This will be the subject of future clinical studies.

Cells can protect themselves against endotoxins and exotoxins. Previous research provides information on DNA proteins and exacerbation-related genes and proteins in COPD.^{2,25,26} As this is the first published study to evaluate AgNOR proteins in patients with COPD exacerbation, there are limited data to compare these current findings with. Some studies that have investigated the role of AgNOR proteins in acute and chronic hypoxia in CO intoxication modelling suggest that increasing AgNOR proteins may play a protective role against hypoxic damage in the cell up to a particular level/duration of exposure.^{21,27} These current findings suggest that AgNOR proteins could be produced on an industrial scale so that they could be utilized in hypoxic/hypercapnic patients. The target patient group may range from acquired hypoxic sequelae of cardiovascular, traumatic or toxic origin to hypoxic asphyxiated delivery of newborns. Further prospective clinical studies are essential to investigate the possible protective potential of AgNOR proteins.

This study had several limitations. First, it had a comparatively low number of

patients. Even though the number of analysed cells ($n=1000$) was enough to understand the changes in AgNOR protein staining in response to hypercapnia, studies with larger numbers of patients should be undertaken. Secondly, no further examinations were undertaken during hospitalization or after discharge. Therefore, it was not possible to compare the AgNOR parameters at baseline with those post-treatment, which would have allowed for a more complete understanding of what happens to AgNORs during COPD exacerbation and treatment. Prospective clinical follow-up studies on patients with COPD exacerbation should be undertaken to establish the relationship between AgNOR proteins and hypercapnia. To better understand the pathophysiology of COPD exacerbation, gene expression studies to isolate AgNOR proteins specific for COPD exacerbation will be essential.

In conclusion, the present study evaluated the clinical application of measuring AgNOR parameters in patients experiencing a COPD exacerbation. AgNOR protein levels were elevated during a COPD exacerbation compared with healthy control subjects and there was a positive correlation between pCO_2 levels and mean AgNOR number. AgNOR parameters may offer a novel technique to identify COPD patients with a risk of poor outcomes.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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