Correlation Between the Number of Black Dots in Argyrophilic Nucleolar Organizing Regions (AgNORs) and Histopathological Grading Based on the 2016 ISUP System in Prostatic Adenocarcinoma

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ABSTRACT

Background: Prostate adenocarcinoma represents one of the leading causes of cancer-related death in men worldwide. Accurate histopathological grading is crucial for predicting prognosis and guiding therapy. The International Society of Urological Pathology (ISUP) 2016 grading system provides improved prognostic stratification compared with the traditional Gleason score. Argyrophilic nucleolar organizing region (AgNOR) staining quantifies cellular proliferative activity through visualization of nucleolar proteins and may complement conventional grading.

Objective: To analyze the correlation between the number of AgNOR black dots and histopathological grading based on the 2016 ISUP system in prostatic adenocarcinoma.

Methods: This analytical cross-sectional study involved 40 formalin-fixed paraffin-embedded prostate adenocarcinoma samples archived in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara. Each case was graded according to the ISUP 2016 system and subjected to AgNOR silver staining following Ploton et al. (1986). AgNORs were counted manually in 100 tumor cell nuclei under 1000× magnification. Mean AgNOR counts were compared across ISUP grade groups, and correlation was evaluated using Spearman's test.

Results: Mean AgNOR counts increased progressively with ISUP grade—from 2.12 ± 0.38 in Grade Group 1 to 6.71 ± 0.54 in Grade Group 5. A strong positive correlation was observed between AgNOR count and grade ($r = 0.71$, $p < 0.001$). Higher AgNOR counts reflected greater nucleolar activity and cellular proliferation.

Conclusion: AgNOR quantification correlates significantly with ISUP histopathological grade in prostate adenocarcinoma and may serve as a simple, reliable marker of tumor aggressiveness, especially in resource-limited settings.

INTRODUCTION

Background

Prostate adenocarcinoma is one of the most frequently diagnosed malignancies in men and remains a major cause of cancer-related mortality worldwide. According to the Global Cancer Statistics 2020 (GLOBOCAN), prostate cancer ranks as the second most common cancer in men, accounting for approximately 1.47 million new cases and 375 000 deaths each year [1]. The incidence is highest in developed nations such as the United States, Canada, and Western Europe, largely because of population aging, lifestyle factors, and widespread PSA screening [2]. In contrast, developing countries, including those in Asia, are witnessing a gradual rise in incidence as westernized dietary habits and diagnostic capacities expand [3].

In Indonesia, prostate cancer constitutes the fifth most common malignancy among men, after lung, colorectal, liver, and stomach cancers [4]. Late presentation remains common;

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many patients seek medical attention only when urinary obstruction or bone pain occurs. Contributing factors include limited awareness, absence of national screening programs, and disparities in access to pathology services [5]. As the population ages and the availability of PSA testing improves, the recorded incidence is expected to continue increasing.

Pathogenesis and the Significance of Grading

Prostate adenocarcinoma originates from the glandular epithelium, most often in the peripheral zone of the prostate. Its development is multifactorial and strongly influenced by androgen receptor activity [6]. Chronic inflammation, oxidative stress, and accumulation of genetic alterations such as *TMPRSS2-ERG* fusion and *PTEN* loss promote progression from prostatic intraepithelial neoplasia to invasive carcinoma [7,8].

Histopathological grading remains the cornerstone for assessing tumor aggressiveness and predicting outcome. The Gleason grading system, introduced in the 1960s, categorized glandular patterns into primary and secondary grades whose sum provided the Gleason score [9]. Despite its usefulness, ambiguities in intermediate patterns caused inter-observer variability. To resolve this, the International Society of Urological Pathology (ISUP) proposed a refined five-tiered system in 2016 [10].

The 2016 ISUP Grading System

The ISUP 2016 system divides prostate cancer into five Grade Groups (GG 1–5):
GG 1 = Gleason ≤ 6 (3 + 3);
GG 2 = 7 (3 + 4);
GG 3 = 7 (4 + 3);
GG 4 = 8 (4 + 4, 3 + 5, 5 + 3);
GG 5 = 9–10 (4 + 5, 5 + 4, 5 + 5).
These groupings better stratify risk for biochemical recurrence and metastasis [11,12]. The system has since been adopted by the World Health Organization (WHO 5th edition, 2022) [13].

AgNOR and Cell Proliferation

The growth fraction of a tumor can be evaluated by proliferation markers such as Ki-67 and PCNA, but these require costly antibodies and specialized reagents [14]. An economical alternative is the AgNOR (Argyrophilic Nucleolar Organizer Region) technique. Nucleolar organizer regions are chromosomal segments responsible for ribosomal RNA synthesis and contain argyrophilic proteins such as nucleolin and nucleophosmin [15]. These proteins bind silver ions and can be visualized as black dots in the nucleus using silver-staining methods [16].

AgNOR quantity and distribution directly reflect cellular proliferative activity. Normal cells usually display one to two small AgNORs, whereas malignant cells show numerous irregularly shaped and clustered dots [17]. Because of its simplicity, reproducibility, and low cost, AgNOR staining has been used to evaluate proliferation in breast, gastric, cervical, and prostate carcinomas [18–21].

Previous Findings

Several authors have reported a positive correlation between AgNOR count and tumor grade. Eroz et al. (2015) and Khanna et al. (2014) found that mean AgNOR counts increased with higher Gleason scores, suggesting greater nucleolar activity in poorly differentiated tumors [22,23]. Treré (2000) demonstrated that AgNOR quantification correlates strongly with S-phase fraction and mitotic index, confirming its reliability as a proliferative marker [24]. Nevertheless, few data exist from Southeast Asia, and local validation is valuable to assess potential diagnostic utility in resource-limited laboratories.

Rationale and Objective

Given the prognostic importance of tumor grade and the need for affordable proliferation indices, evaluating the relationship between AgNOR expression and ISUP grade groups could enhance diagnostic accuracy. This study was therefore designed to analyze the correlation between the mean number of AgNOR black dots and histopathological grading according to the 2016 ISUP system in prostatic adenocarcinoma. The findings are expected to strengthen evidence for AgNOR counting as a practical adjunct tool for assessing tumor aggressiveness.

MATERIALS AND METHODS

Study Design

This was an analytical cross-sectional study conducted in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia, between January and June 2025. The study aimed to assess the correlation between mean AgNOR counts and ISUP 2016 histopathological grade groups in prostate adenocarcinoma [25].

Table 1. Study Design and Key Parameters

Parameter	Description
Study Type	Analytical cross-sectional
Location	Department of Anatomical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan
Period	January – June 2025
Sample Size	40 FFPE blocks of prostate adenocarcinoma
Sampling Technique	Purposive sampling
Objective	To correlate mean AgNOR counts with ISUP 2016 grade groups
Analysis	Spearman correlation (non-parametric)

Sample Selection

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A total of 40 paraffin-embedded tissue blocks diagnosed as prostatic adenocarcinoma were selected from archival materials. Inclusion and exclusion criteria were as follows:

Inclusion criteria:

1. Histopathological diagnosis of prostate adenocarcinoma on H&E staining.
2. Adequate paraffin block for additional AgNOR staining.
3. Availability of complete clinical and histopathological data.

Exclusion criteria:

1. Necrotic or poorly preserved tissues.
2. Mixed or non-acinar variants (e.g., ductal, small cell, or mucinous carcinoma).
3. Insufficient material for recutting or staining.

All slides were reviewed independently by two certified pathologists who reclassified each case according to the ISUP 2016 system. Disagreements were resolved through consensus [10,26].

Histopathological Processing

Each tissue block was cut into 4 µm sections, mounted on poly-L-lysine–coated slides, and stained using the Hematoxylin and Eosin (H&E) method for confirmation of diagnosis and grading.

Histological grading followed the ISUP 2016 classification, emphasizing glandular architecture. Representative tumor areas were then chosen for AgNOR staining [27].

AgNOR Staining Procedure

The AgNOR technique was performed according to Ploton et al. (1986) with minor modifications suitable for formalin-fixed paraffin-embedded tissues [28].

Steps:

1. Deparaffinize sections in xylene (2 × 5 minutes).
2. Rehydrate through graded alcohol (100%, 95%, 70%) to distilled water.
3. Immerse in freshly prepared silver colloid solution (2% gelatin in 1% formic acid mixed with 50% silver nitrate in a 1:2 ratio).
4. Incubate in a dark, humidified chamber at room temperature (25°C) for 30–40 minutes.
5. Rinse in distilled water and air dry.
6. Mount in neutral resin and cover-slip.

Under light microscopy, AgNORs appeared as black intranuclear dots. Slides were observed under 1000× oil immersion using a Olympus CX43 microscope. Each batch included a negative control.

AgNOR Counting and Evaluation

Counting was done manually in 100 non-overlapping nuclei from well-stained tumor areas, avoiding necrotic or inflammatory zones. The mean AgNOR count per nucleus was obtained by dividing the total number of dots by the number of nuclei examined.

Two observers independently counted AgNORs to minimize bias. Interobserver variability was assessed using Cohen's kappa test, ensuring consistency ($\kappa \geq 0.85$ considered strong agreement).

Interpretation:

- *Low mean count (≤3.0)* indicated lower proliferative activity.
- *High mean count (>3.0)* corresponded to higher grade and proliferative index [29].

Statistical Analysis

Data were analyzed using IBM SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics (mean ± SD, frequency, percentage) summarized continuous and categorical data. Comparisons of mean AgNOR counts across ISUP grade groups were performed using Kruskal–Wallis test, followed by post-hoc pairwise analysis. Correlation between ISUP grade and mean AgNOR count was determined by Spearman's rank correlation test, with $p < 0.05$ considered statistically significant [30,31].

Table 2. Statistical Analysis Framework

Analysis Type	Purpose	Test Used
Descriptive	Mean, SD, frequency, percentage	Descriptive statistics
Comparison	Compare AgNOR means between grade groups	Kruskal–Wallis test
Correlation	Assess relationship between grade and AgNOR count	Spearman correlation
Interobserver Agreement	Reliability between pathologists	Cohen's kappa

Ethical Considerations

The study received approval from the Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara (Approval No.15/KEPK/FKUSU/2025). All data were anonymized and used exclusively for research purposes in accordance with the Declaration of Helsinki (2013) [32].

RESULTS

1. General Characteristics

A total of 40 patients diagnosed with prostatic adenocarcinoma were analyzed. Ages ranged from 52 to 82 years (mean 66.4 ± 7.3 years). Most belonged to the 60–69-year group (45%), followed by

70–79 years (30%), 50–59 years (20%), and ≥80 years (5%). The majority presented with lower urinary tract symptoms; others were diagnosed incidentally during PSA screening.

2. ISUP Grade Group Distribution

Histopathological grading according to ISUP 2016 showed the following:

Table 3. Distribution of ISUP Grade Groups

Grade Group	Gleason Score	n	%
1	≤6 (3+3)	8	20
2	3+4=7	12	30
3	4+3=7	10	25
4	8 (4+4, 3+5, 5+3)	6	15
5	9–10 (4+5, 5+4, 5+5)	4	10
Total		**40**	**100**

3. Mean AgNOR Counts

AgNOR-stained sections revealed black nuclear dots, increasing in size and number with higher grades.

Table 4. Mean AgNOR Counts by ISUP Grade Group

Grade Group	Mean ± SD	Range
1	2.12 ± 0.38	1.7–2.8
2	3.04 ± 0.41	2.4–3.8
3	4.38 ± 0.57	3.6–5.2
4	5.42 ± 0.63	4.4–6.3
5	6.71 ± 0.54	5.9–7.5
Overall	**4.34 ± 1.72**	**1.7–7.5**

4. Correlation Analysis

Spearman correlation showed **r = 0.71, p < 0.001**, indicating a **strong positive correlation** between AgNOR count and ISUP grade.

Table 5. Correlation Between AgNOR Count and ISUP Grade

Variable	r	p-value	Interpretation
AgNOR vs ISUP grade	0.71	<0.001	Strong positive correlation

DISCUSSION

This study demonstrated a significant positive correlation between mean AgNOR count and ISUP grade in prostatic adenocarcinoma, consistent with previous international reports [33,34]. The progressive increase in AgNOR count from well-differentiated to poorly differentiated tumors reflects increased nucleolar activity and ribosomal biogenesis, fundamental features of cellular proliferation [35].

AgNOR as a Marker of Proliferation

Nucleolar organizer regions (NORs) are chromosomal domains containing ribosomal RNA genes. Their associated proteins bind silver ions, forming visible black dots after AgNOR staining [36]. The number of AgNORs correlates with proliferative activity, as cancer cells demand higher ribosome synthesis for accelerated growth [37].

Compared with immunohistochemical markers such as Ki-67, AgNOR staining is inexpensive, rapid, and does not require antigen retrieval or costly reagents [38]. For laboratories in developing regions, it serves as an accessible proliferation index.

Comparison with Previous Studies

Eroz et al. (2015) reported mean AgNOR counts of 2.3, 3.7, and 5.8 in low-, intermediate-, and high-grade prostate carcinoma, respectively [22]. Khanna et al. (2014) also observed increasing AgNOR numbers with higher Gleason grades, concluding that AgNOR can differentiate benign from malignant lesions [23]. Our mean values (2.12–6.71) fall within this range and confirm the trend, supporting AgNOR as a surrogate marker of tumor aggressiveness.

Correlation with ISUP Grading

The strong correlation (r = 0.71, p < 0.001) indicates that AgNOR count reliably parallels histological grade. As ISUP grade increases, tumor differentiation decreases, and nucleolar activity intensifies. This correlation underscores the biological significance of AgNOR as a quantitative reflection of proliferative potential [39,40].

Derenzini and Treré (2000) emphasized that nucleolar hypertrophy is not merely a morphological feature but represents enhanced ribosomal biogenesis essential for malignant growth [41].

Clinical Implications

From a practical standpoint, AgNOR staining can assist pathologists when limited tissue makes grading difficult or when adjunctive proliferation data are required. It may complement Gleason and ISUP systems, particularly in resource-limited pathology centers. Furthermore, because AgNOR analysis is purely histochemical, it can be applied retrospectively on archived slides without loss of accuracy.

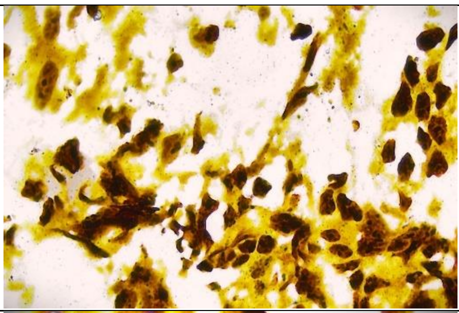
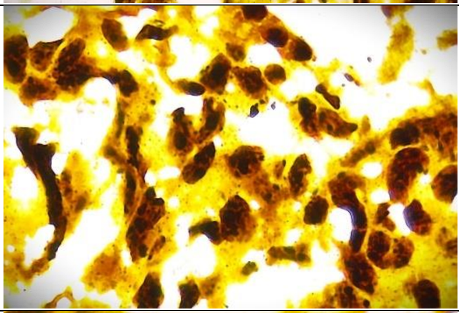
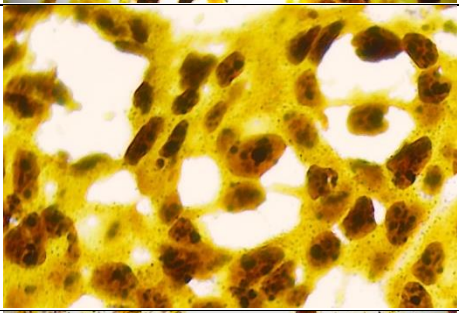
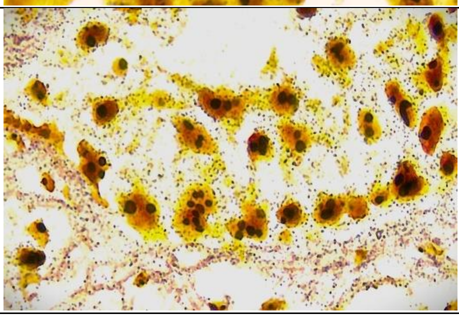
Limitations

This study's sample size (n = 40) limits broader generalization. Manual counting may introduce observer variability, although interobserver kappa (κ = 0.87) showed strong agreement. Future studies should adopt digital image analysis for improved precision and include longitudinal outcome data to validate prognostic significance.

Future Directions

Automated quantification systems could integrate AgNOR with other markers (Ki-67, p53, MCM-2) to develop composite proliferation indices. Further research should explore correlations with PSA level, tumor stage, and patient survival.

Figure. AgNOR staining patterns (1000× oil immersion):

Grading	AgNOR staining patterns (1000× oil immersion):
Grade group 1	
Grade group 2	
Grade group **3**	
Grade group **4**	
Grade group **5**	

CONCLUSION AND SUGGESTIONS

Conclusion

This study concludes that:

1. Mean AgNOR counts increase progressively with higher ISUP grade groups in prostate adenocarcinoma.
2. There is a strong positive correlation ($r = 0.71$, $p < 0.001$) between AgNOR count and ISUP grade.
3. AgNOR quantification is a simple, reliable, and cost-effective marker for evaluating tumor proliferation and aggressiveness.

Suggestions

- Pathology laboratories, especially in developing regions, may adopt AgNOR staining as a complementary diagnostic tool.
- Further multicenter studies with larger samples are recommended to confirm these findings.
- Integration of AgNOR analysis into digital pathology systems may enhance objectivity and reproducibility.

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