Role of cyclin D1 immunoreactivity and AgNOR staining in the evaluation of benign and malignant lesions of the prostate

Veena Gupta, Monika Garg, Manish Chaudhry, Sunita Singh, Rajeev Sen, Meenu Gill, Ashok Sangwaiya

Department of Pathology, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Rohtak, India
1Department of Pathology, MM Institute of Medical Sciences & Research, Mullana, India

Purpose: Prostatic carcinoma is a common and growing public health problem. Histological evaluation is fairly adequate for assessing tumor differentiation, but tumor proliferative activity is difficult to measure. Increasing evidence suggests that the factors controlling cell cycle progression also modulate the rate of ribosome biogenesis. Despite the influence of cyclin D1 and argyrophilic nuclear organizer region (AgNOR) on prostate cancer proliferation, few studies have evaluated the diagnostic importance of these markers. Therefore, the present study was carried out to analyze the diagnostic value of the proliferative markers cyclin D1 and AgNOR in various prostatic lesions and to determine whether any association or relation between these markers and different Gleason grades exists.

Methods: A total 50 cases of various prostatic lesions were studied. Tumor grade, AgNOR staining, and cyclin D1 expression were evaluated in all cases. Correlations between the intensity and differential localization of these markers and Gleason grades were evaluated.

Results: The mean AgNOR count in cases of prostatic intraepithelial neoplasia was high compared with cases of benign prostatic hyperplasia (BPH) but lower than that of carcinoma cases. The intensity of cyclin D1 expression was high in carcinoma. A total of 14 cases (46.67%) showed strong positivity. No significant correlation was found between the intensity of cyclin D1 expression, AgNOR count, and histologic grades of prostatic carcinoma, whereas a significant correlation was observed between intensity and percentage expression of cyclin D1 in BPH and carcinoma (P < 0.01). Nuclear as well as cytoplasmic positivity was seen among various grades of carcinoma.

Conclusions: AgNOR count and cyclin D1 may be helpful in distinguishing between BPH and carcinoma of the prostate but may not be used as reliable indicators of the grade of prostatic adenocarcinoma because of overlapping values in various grades. However, further studies on larger samples are required to elucidate the role of these markers in identification of premalignant lesions.

Keywords: Prostate, Carcinoma, Prostatic hyperplasia, Cyclin D1, AgNORs, Immunohistochemistry

INTRODUCTION

Prostatic disease is responsible for significant morbidity and mortality in men throughout the world. The diagnosis of prostate adenocarcinoma is often challenging. Whereas histological evaluation is fairly adequate for assessing differentiation, tumor proliferative activity is difficult to measure. The identification of molecular events underlying cell transformation may expand our understanding of the natural history of the disease. Nucleolar organizer regions (NORs) are segments of metaphase chromosomes where ribosomal genes are located and which correspond to secondary constrictions.
These structures contain all necessary components for rRNA synthesis and are the sites where the transcription of ribosomal genes occurs [2]. The silver-stained NORs are called argyrophilic nucleolar organizer region associated proteins (AgNORs). AgNORs in normal cells are usually tightly aggregated within one or two nuclei, thus making individual Ag-NORs indiscernible. An increase in the mean AgNOR count of a cell population could be the result of a defect in nucleolar aggregation, association, ploidy, or increased transcriptional activity [3,4]. Theoretically, a neoplastic cell population could show any or all of the above defects and therefore demonstrate an increased AgNOR count. Several studies have shown that AgNOR frequency within nuclei is significantly higher in malignant cells than in normal, reactive, or benign neoplastic cells. AgNOR expression is directly related to the ribosome biosynthesis rate, which, in proliferating cells, is directly related to the length of the cell cycle. The shorter the cell cycle, the greater the synthesis of rRNA for each time unit and, therefore, the greater the quantity of AgNOR present in the nucleolus. Thus, the AgNOR value was thought to be a measure of the rate of cell proliferation [1].

Cyclin D1 is an amino acid that is expressed in the G1 phase of the cell cycle and that has an important role in regulating the cell cycle and cancer progression. Its over-expression is believed to play an important role in both the tumorigenesis and grading of many cancers, including prostatic carcinoma. There is increasing evidence that the factors controlling cell cycle progression also modulate the rate of ribosome biogenesis. In prostatic cancer, cyclin D1 acts as a critical regulator of androgen-dependent transcription and cell cycle progression [5].

Despite the influence of cyclin D1 and AgNOR on prostate cancer proliferation, few studies have evaluated the diagnostic importance of these markers. Therefore, the present study was carried out to analyze the diagnostic value of the proliferative markers cyclin D1 and AgNOR in benign and malignant lesions of the prostate and also to prove or disprove an association or relation between these markers and different Gleason grades.

**MATERIALS AND METHODS**

Clinical and pathological data: This prospective study was carried out in the Department of Pathology, Pandit Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences, Rohtak, India. A total of 50 cases of prostatic biopsies were taken for study. Brief clinical data were noted from case records.

Histopathological evaluation: All prostatic specimens were subjected to careful and detailed gross examination. Tissue sections were fixed in 10% formalin and embedded in paraffin and were used for microscopic study. Sections 4 to 5 µ thick were prepared and stained routinely with hematoxylin and eosin (H&E) [6]. These sections were then studied under a light microscope and were classified as benign or malignant lesions. Special stains like van Gieson, periodic acid-Schiff, Masson’s trichrome, and reticulin were used whenever required for histopathological diagnosis.

Silver staining for AgNOR and immunohistochemistry: AgNOR staining was performed according to the guidelines of the “International Committee on AgNOR Quantitation” [7]. AgNOR was observed and counted as black dots in the nuclei of at least 100 cells by using a 100× oil immersion objective and the average was calculated [8].

Immunohistochemistry was performed by the peroxidase-antiperoxidase method [9]. Positive and negative controls were run simultaneously. Tissue from the tonsil (epithelium) was used as a positive control. A negative control was made by substituting primary antibody with antibody of irrelevant specificity.

Immunohistochemistry evaluation: Cyclin D1 staining intensity was assessed as follows [10]:

- 0 (absent)
- 1– (weak/focal < 10% of sample)
- 1+ (weak intensity < 25% of sample)
- 2+ (moderate intensity 25%–50% of sample)
- 3+ (strong intensity > 50% of sample)

The data thus obtained were analyzed in the form of mean AgNOR count/cell and cyclin D1 expression in all benign and malignant cases including various grades of prostatic carcinoma. Cyclin D1 was assessed in the form of percentage of expression and staining intensity and was scored as 0, 1–, 1+, 2+, and 3+ as well as by the pattern of expression whether nuclear, cytoplasmic, or both [11,12].

Statistical analysis: The results thus obtained were interpreted and correlated statistically. Comparison between multiple groups was made by using Student’s t-test, chi-square test, and analysis of variance test, whichever was appropriate. A value of $P < 0.05$ was taken as significant and $< 0.01$ was taken as highly significant, whereas $P$-values of $> 0.05$ were taken as nonsignificant.

**RESULTS**

A total of 50 cases were used in the present study, which in-
cluded 18 cases (36%) of benign prostatic hyperplasia (BPH), 2 cases (4%) of prostatic intraepithelial neoplasia (PIN), and 30 cases (60%) of carcinoma. The majority of specimens were collected by needle biopsy (68%), followed by transurethral resection of the prostate (24%) and prostatectomy (8%). The age of the patients ranged from 46 to 95 years with a class interval of 5 years and a mean age of 71.3 ± 10.16 years. The majority of cases (11 cases) were in the age group of 76–80 years, which formed 22% of the study group. Patients with carcinoma of the prostate were in the age group of 51–95 years with a mean age of 72.43+10.49 years. The maximum number of cases was in the age group of 76 to 80 years (7 cases), followed by the age group of 61–65 years (5 cases).

All foci of PIN were of high-grade PIN. The Gleason score was estimated in all cases by taking the primary grade (most prevalent) into consideration. Among the 30 cases of prostatic carcinoma, 17 cases (56.66%) were of intermediate grade, 9 cases (30.0%) were of high grade, and only 4 cases (13.33%) were of low grade.

1. **AgNOR quantitative distribution**

   The range of AgNOR dots per nucleus in BPH was 1.80–4.68 with a mean of 3.03 ± 1.02. In cases of PIN, the range was 4.12–4.96 with a mean of 4.54 ± 0.59. The corresponding figures for carcinoma were 2.06–7.74 with a mean of 5.37 ± 1.31.

   The difference in mean number of AgNOR dots per nucleus between cases of BPH and cases of carcinoma was statistically significant (P<0.001). The mean AgNOR count in cases of PIN was high compared with cases of BPH but was lower than in carcinoma cases. However, a statistical correlation could not be calculated because of the small number of cases.

   The range of AgNOR dots per nucleus in cases of low-grade carcinoma was 4.36–5.92 with a mean of 4.97 ± 0.67. On the other hand, the range in intermediate-grade carcinoma was 2.06–7.74 with a mean of 5.25 ± 1.57. The range in high-grade carcinoma was 4.32–7.24 with a mean of 5.78 ± 0.94. The difference in mean number of AgNOR dots per nucleus between the three grades of carcinoma was statistically insignificant.

2. **Cyclin D1 status**

   Cyclin D1 expression was assessed in various prostatic lesions by use of the peroxidase antiperoxidase immunohistochemical method. Nuclear and cytoplasmic staining intensity was assessed and scored semi quantitatively. There was no expression of cyclin D1 in 5 cases (27.77%) of BPH, whereas in PIN and carcinoma it was expressed in 100% of cases. There was a statistically significant difference in expression of cyclin D1 between BPH and carcinoma cases, as indicated by a P-value of <0.01. Of the BPH cases, the maximum number of cases (7) showed focal positivity (38.88%), followed by absence of positivity and weak positivity (5 cases each). One case showed moderate positivity. No case showed strong positivity. Among two cases of PIN, one showed moderate positivity and the other showed strong positivity.

   Of the carcinoma cases, the maximum number of cases (14, or 46.67%) showed strong positivity, followed by moderate positivity in 10 cases (33.33%), weak positivity in 5 cases (16.67%), and focal positivity in 1 case (3.33%). Absence of cyclin D1 expression was not noted in any case.

   There was a statistically significant difference in intensity and percentage of expression of cyclin D1 between BPH and carcinoma (P<0.01). The correlation of cyclin D1 expression with histologic grading in prostatic carcinoma is presented in Table 1.

3. **Altered cyclin D1 expression**

   Of the BPH cases, 13 showed cyclin D1 expression, of which 8 cases showed only nuclear positivity and 5 cases showed both nuclear and cytoplasmic positivity. Both cases of PIN showed nuclear as well as cytoplasmic positivity. Of the carcinoma cases, 24 cases showed both nuclear and cytoplasmic positivity, whereas 2 cases showed cytoplasmic and 4 cases showed nuclear positivity only. The localization of cyclin D1 expression in various grades of prostatic carcinoma is shown in Table 2 and Figs. 1–4.

### DISCUSSION

Prostate cancer is an important growing health problem with an often unpredictable course that presents a diagnostic challenge to urologists, radiologists, and pathologists. The tumor is dependent on androgen for growth and survival. Androgen elicits its biological effect through activation of the androgen receptor. Upon ligand binding, the activated receptor stimulates a gene expression program that induces cellular proliferation. Androgen induces accumulation of D type cyclins,
which interact with and activate cyclin-dependent kinases to promote G1 progression during the cell cycle [10,13,14]. Tumor differentiation and proliferative activity are important predictors of biological behavior. Routine histopathological evaluation is fairly adequate for assessing differentiation. Tumor proliferative activity is difficult to measure, however. Silver staining for the NOR is reported to be useful for assessing tumor proliferation. The present study was planned to analyze the AgNOR count and cyclin D1 expression in various prostatic lesions and to assess whether these can be useful in differentiating benign from malignant lesions and various grades of prostatic carcinomas. The study was also done to evaluate any correlation between the two markers.

In prostate lesions, many studies have shown a significantly increased proportion of proliferating cells in carcinoma compared with benign lesions, with the greatest proliferative indexes noted in high-grade carcinomas [15]. Thus, AgNOR counts increase with the aggressiveness of the tumor and are highest in high-grade prostatic carcinomas. In the present study, an increase in mean AgNOR count was noted from BPH to PIN and carcinomas. Furthermore, the mean AgNOR counts of carcinomas were significantly different from those of BPH. Similar observations were reported in other previous

**Table 2.** Localisation of cyclin D1 expression in various grades of prostatic carcinoma

| Grade                  | Nuclear only | Cytoplasmic only | Both nuclear and cytoplasmic |
|------------------------|-------------|------------------|-----------------------------|
| Low grade (n=4)        | 0           | 0                | 4                           |
| Intermediate grade (n=17)| 2          | 2                | 13                          |
| High grade (n=9)       | 2           | 0                | 7                           |

**Fig. 1.** Photomicrograph of benign prostatic hyperplasia specimen (H&E, ×200). Inset shows negative cyclin D1 expression and 4 to 5 AgNOR dots per nucleus.

**Fig. 2.** Photomicrograph of prostatic intraepithelial neoplasia specimen (H&E, ×200). Inset shows moderate cyclin D1 positivity and 2 to 4 AgNOR dots per nucleus.

**Fig. 3.** Photomicrograph of high-grade carcinoma specimen showing strong cyclin D1 positivity with inset revealing perineural invasion (immunohistochemistry, ×200).

**Fig. 4.** Photomicrograph of high-grade carcinoma specimen revealing 5 to 7 AgNOR dots per nucleus (AgNOR, ×1,000).
studies [3,4].

Hansen and Ostergard [3], however, reported overlapping counts. They concluded that, despite statistically significant differences, AgNOR counts are of no use for diagnosis of any single case. AgNOR typing, however, may contribute to a differential diagnosis between benign and malignant lesions. Chiusa et al. [16] concluded that AgNOR counts not only reflect cell proliferation but also the degree of cellular differentiation. Therefore, a combination of histological grading and AgNOR counts allows the stratification of low- and high-risk groups.

Ghazizadeh et al. [17] also observed that the mean AgNOR count significantly increases with grade and clinical stage of tumor. They concluded that AgNOR counting may contribute to making a conventional diagnosis and prognosis of carcinoma of the prostate. In our study, we observed overlapping AgNOR counts in various grades of carcinoma of the prostate, and the difference was not statistically significant.

Ahsan et al. [18] also observed a significantly different count in nodular hyperplasia and carcinoma. They concluded that the AgNOR technique is rapid, simple, and reproducible, although somewhat tedious and laborious. It can differentiate between nodular hyperplasia and carcinoma of the prostate on the basis of AgNOR count per cell. However, a small overlap in the range of counts along with the absence of a clear-cut gap between the counts in the two groups somewhat limits the importance of simple enumeration of AgNOR in an isolated case of nodular hyperplasia or carcinoma of the prostate.

Cyclin D1, a cell regulatory protein that is considered a product of the cyclin D1 protooncogene, is an important regulator of the G1 to S phase transition of the cell cycle. It is believed to play an important role in both tumorigenesis and grading of many cancers, including prostatic carcinoma, if its expression is deregulated, mainly overexpressed [19]. Chen et al. reported that overexpression of cyclin D1 increases cell growth and tumorigenicity in human prostate cancer [20].

Several studies have been carried out in the past to see the expression of cyclin D1 in prostatic lesions. It has been observed that cyclin D1 expression is enhanced in most localized tumors compared with nonneoplastic epithelium, thus indicating that cyclin D1 is aberrantly regulated in prostate cancers. It has previously been shown through in vitro studies that cyclin D1 can influence androgen-dependent prostate cancer cell proliferation through its dual ability to modulate both CDK 4 and androgen receptor activity [10].

Drobnjak et al. [21] concluded that cyclin D1 expression along with the proliferative index are associated with the clinicopathological parameter of poor clinical outcome. However, no correlation was observed between cyclin D1 overexpression and either Gleason’s score, neo-adjuvant hormone treatment, or PSA relapse.

In 2001 Ueda et al. [22] found that 53.8% of cases of BPH and 84.6% of cases of prostatic carcinoma showed cyclin D1 expression. These results indicate that cyclin D1 expression tends to increase in malignant prostate tissue. Fleischmann et al. [23] in 2010 found that in primary tumors high nuclear cyclin D1 expression was significantly correlated with poor tumor differentiation and large nodal tumor burden. In the present study, we studied the expression of cyclin D1 in 50 prostate samples. There was no expression of cyclin D1 in 5 cases (27.77%) of BPH, whereas in PIN and carcinoma it was expressed in 100% of cases.

Our findings are in concordance with the results of previous studies indicating that cyclin D1 expression is seen with higher frequency in prostatic carcinoma (Table 3). We also observed that focal and weak staining may be seen in benign cases but that it never reaches a significant proportion as seen in carcinoma of the prostate. However, we observed no significant correlation of cyclin D1 expression with Gleason grade, which is similar to some studies [22,24] but in contrast with others [10,25]. The difference in the findings may be explained on the basis that most studies focused largely on nuclear cyclin D1, but common conclusions failed to emerge.

In the present study, we also observed different patterns of

| Study | No. of cases | Cyclin D1 positivity | Correlation |
|-------|--------------|----------------------|-------------|
|       |              | BPH | Carcinoma | BPH | Carcinoma |                          |
| Shiraishi et al. [24] | - | 66 | - | 20 (30.3) | Absent |
| Drobnjak et al. [21] | - | 86 | - | 10 (11.6) | Absent |
| Ozbek et al. [25] | 25 | 30 | * | 30 (100) | Present |
| Ueda et al. [22] | 26 | 13 | 14 (53.8) | 11 (84.6) | - |
| Comstock et al. [10] | 23 | 36 | 3 (13.0) | 34 (94.4) | Present |
| Present study | 18 | 30 | 13 (72.2) | 30 (100) | Absent |

Values are presented as number (%) unless otherwise indicated.

*Correlation of cyclin D1 positivity with histologic grade. *Weak stromal staining.
cyclin D1 expression. Comstock et al. [10] also found low or absent cyclin D1 expression in nonneoplastic tissue and increased expression in most localized tumors. They observed different patterns of cyclin D1 expression in the tumor. Most tumors showed cytoplasmic restriction of cyclin D1, whereas high-grade tumors showed nuclear cyclin D1 staining. They concluded that cyclin D1 can be differentially expressed in prostatic cancer and that the status or localization of cyclin D1 expression is associated with meaningful changes in tumor marker expression and proliferative indices [21].

In conclusion, BPH and adenocarcinoma are common diseases that account for considerable morbidity and mortality in the aging population. AgNOR count and cyclin D1 may be helpful in distinguishing between BPH and carcinoma of the prostate but may not be used as a reliable indicator of grading prostatic adenocarcinoma because of overlapping of values in various grades. However, further studies on larger samples are required to elucidate the role of these markers in the identification of premalignant lesions. Also, the significance of differential expression and localization of cyclin D1 needs to be evaluated further. There is a need for research on all aspects of this disease. The ultimate conquest of prostatic carcinoma will require substantial advances in our understanding of the cause of this tumor, further development and refinement of diagnostic techniques, and development of new therapeutic modalities for treatment of systemic disease. We can look forward to further exciting developments in this important area of cancer cell research in the coming years.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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