Original Article

Patterns of nucleolar organizer region in urinary bladder cancer in view of 2004 WHO classification of urothelial neoplasms

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ABSTRACT

Context: The last decade has seen a tremendous upheaval since introduction of WHO-1998 classification (modified in 2004) of bladder carcinoma, which only recently seems to have settled down. We compare both old (WHO-1973) and new (2004) classifications with argyrophilic nucleolar organizer region (AgNOR) score. **Aims:** To compare WHO/ISUP (World Health Organization/International Society of Urologic Pathologists) consensus classification-2004 and WHO-1973 classification of bladder carcinoma and to establish correlation between AgNOR scores and various grades of carcinoma bladder. **Materials and Methods:** Sixty H and E (Hematoxylin and Eosin) stained sections of transitional cell carcinoma bladder were graded according to WHO/ISUP (2004) and WHO (1973) classification systems and the results were compared. This was then followed by AgNOR staining of the paraffin-embedded sections and subsequent AgNOR scores were calculated and given as total score, extranucleolar score, intranucleolar score, and nucleolar score. These were correlated with the grade of the tumor. **Statistical Analysis Used:** ANOVA test. **Results:** It was observed that the two grading systems, WHO/ISUP (2004) and WHO (1973) correlated with each other. Also it was observed that the total AgNOR score and extranucleolar AgNOR score increased with the grade of the tumor and thus increasing scores carry significance in assessment of the grade of the tumor. However, intranucleolar and nucleolar scores showed variable degree of overlap in various grades of the bladder tumors. **Conclusions:** WHO/ISUP (2004) classification correlated well with the WHO (1973) classification. The total AgNOR score was found to be helpful in assessment of the grade of the tumor but the distribution of the AgNOR carried little significance.

Key words: Argyrophilic nucleolar organizer region score, urothelial neoplasms, 2004 WHO classification

INTRODUCTION

Attempts toward earliest classification of transitional cell carcinoma (TCC) bladder started as early as 1940s, when Ash proposed the first accepted classification of bladder carcinoma.[1] Since then various classification systems have been proposed, most commonly used being WHO (World Health Organization) -1973 classification and WHO/ISUP (World Health Organization/International Society of Urologic Pathologists)-1998 classification. The latter was essentially reproduced as the 2004 WHO classification scheme.[2] The WHO (1973) classification includes grade I, II, and III, whereas the WHO/ISUP (2004) classification includes papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade lesion and high-grade lesion. PUNLMP, a new entity was introduced to bridge the gap between papilloma and low-grade lesion. This is important owing to increased risk of developing a more aggressive malignant lesion.[2-4]

Further, one of the important proliferation markers, which can be used to assess the tumor aggressiveness, is AgNOR (argyrophilic nucleolar organizer region) score. Nucleolar organizer regions (NOR) have loops of DNA transcribed into rRNA located at each of short arm of the acrocentric chromosome 13,14,15,21, and 22.[5] Various studies have shown that there is an increase in AgNOR score with

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increase in the grade of the tumor owing to increased transcriptional activity. AgNOR score studies on bladder neoplasms have indicated statistical separation between normal and neoplastic urothelium. Moreover, malignant lesions have polymorphic AgNOR dots and tendency toward clumping.[6] However, these studies have been limited by some degree of overlap in various grades of the bladder tumors.[6,7]

The aim of the article was to compare WHO/ISUP (2004) classification of bladder carcinomas with WHO (1973) classification and to establish correlation between AgNOR scores and various grades of bladder carcinomas.

MATERIALS AND METHODS

Sixty cases of TCC bladder diagnosed during January 2005 to February 2010 were included in the study. These cases were obtained from transurethral resection of bladder tumors. Biopsies were processed routinely with paraffin embedding, followed by H and E (Hematoxylin and Eosin) and AgNOR staining. AgNOR staining was done by the following method.[8]

Solutions used for AgNOR staining were:

1. 50% silver nitrate solution (silver nitrate 50 g and distilled water 100 ml)
2. Gelatin solution (gelatin 2 g, formic acid 1 ml and distilled water 100 ml)

Working solution was prepared using 2 parts of silver nitrate solution and 1 part of gelatin solution. The solutions were mixed just before use.

Method used for staining was as below:

- Dewax sections in xylene
- Hydrate through alcohols to water
- Rinse sections in distilled water
- Incubate in freshly prepared working solution for 45 min at room temperature
- Wash in distilled water for 1 minute
- Dehydrate clear and mount in DPX

Grading of the carcinoma was done according to the WHO/ISUP (2004) and WHO (1973) classification. The presence of invasion of lamina propria, muscularis propria, vascular and perineural invasion, squamous metaplasia was also noted. AgNOR scoring was done under oil immersion (100 × objective) on hundred malignant cells, which were then interpreted as extranucleolar, intranucleolar, nucleolar scores.[9] Then a total score of a particular nucleus was obtained by adding these scores. The mean of hundred malignant nuclei was then calculated to obtain AgNOR score of a particular case. The results of the two grading systems were then statistically compared (Chi square test). Analysis Of Variance (ANOVA) test was used for assessment of difference in AgNOR scoring. A p value of less than 0.05 was considered as statistically significant.

RESULTS

The cases belonged to a broad age group ranging from 36 to 85 years with a peak in the age range of 61 to 70 years. The mean age of males was 61.5 years while of females it was 65.3 years. Male to Female ratio was 5:1. Hematuria was seen in 80.2% of cases, whereas obstructive symptoms were seen in 70.4% cases. About 40.8% of the cases were smokers. Other significant etiological factors included history of vesical calculus in 3.3% cases, history of chemical exposure in 9.4% cases (workers in dye industry), and history of radiation therapy for carcinoma cervix in 6.9% cases.

Grading of the carcinoma was done using WHO/ISUP (2004) and WHO (1973) classification [Tables 1 and 2]. The results of which were compared using the chi square test [Table 3]. Invasion of muscularis propria [Figure 1a] was seen in 75% cases of the high-grade lesions. About 10% of cases showed foci of squamous metaplasia. One case showed foci of both vascular and perineural invasion [Figure 1b and 1c]. AgNOR scores were then counted in tumors histologically diagnosed

Table 1: Grading of the cases of transitional cell carcinoma bladder according to WHO/ISUP classification (2004)

WHO/ISUP (2004) Classification	Number of cases (%) n = 60
Papilloma	0 (0)
PUNLMP	16 (26.6)
Low grade	29 (48.3)
High grade	15 (25.0)

Table 2: Grading of the cases of transitional cell carcinoma bladder according to WHO classification (1973)

WHO (1973) Classification	Number of cases (%) n = 60
Papilloma	0 (0)
Grade I	21 (35.0)
Grade II	25 (41.7)
Grade III	14 (23.3)

Table 3: Comparison of WHO/ISUP (2004) with WHO (1973) classification

		WHO (1973) Classification			
		Grade 1	Grade 2	Grade 3	Total
WHO/ISUP (2004) Classification	PUNLMP	16	0	0	16
	Low grade	5	24	0	29
	High grade	0	1	14	15
Total		21	25	14	60

as PUNLMP [Figure 2], low-grade lesions [Figure 3], and high-grade lesions [Figure 1d and 4]. Differences in the scores were assessed using the ANOVA test [Tables 4–8].

The mean and the extranucleolar AgNOR scores increased with the grade of the lesion, whereas the nucleolar and intranucleolar scores showed variable results with increasing grade of the lesion thus concluding that the overall AgNOR scores correlated with the grade of the tumor but the pattern of staining of AgNOR dots does not correlate with the increasing grade of the tumor in bladder carcinoma.

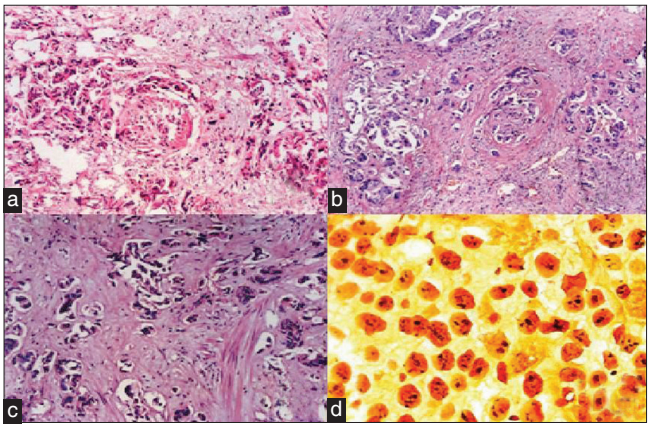

Figure 1: High-grade lesion showing perineural invasion, vascular invasion, and invasion of muscularis propria, (a), (b) and (c), respectively, (H and E, ×400). Also showing typical AgNOR stain (×400) for these high grade lesions (d)

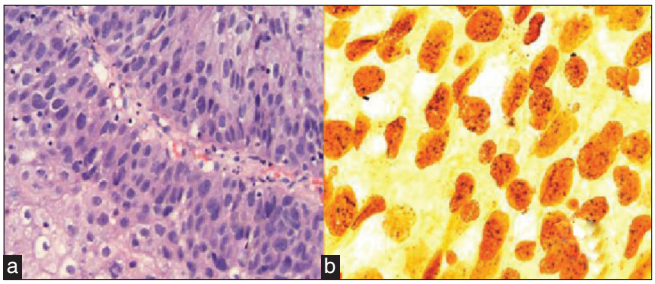

Figure 3: Low-grade lesion (a) (H and E, ×400) and (b) AgNOR stain (×400)

DISCUSSION

TCC accounts for most common malignancies of the urinary bladder.[10] Peak incidence of TCC is seen in sixth decade. McCarthy *et al*.[11] found that only 0.8% of these are present under the age of 30 years. Cases in this study belonged to

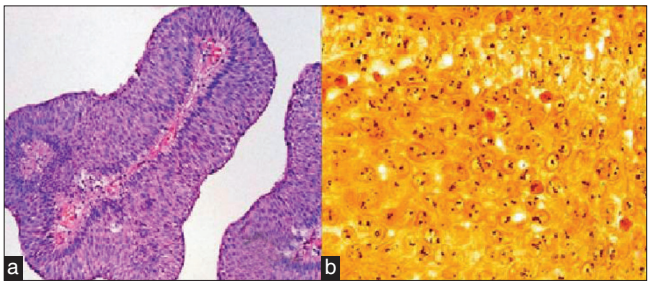

Figure 2: PUNLMP (a) (H and E, ×100) and (b) AgNOR stain (×400)

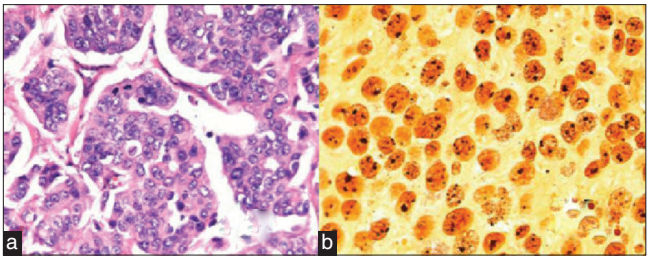

Figure 4: High-grade lesion (a) (H and E, ×400) and (b) AgNOR stain (×400) (×400)

Table 4: Mean scores (nucleolar) and standard deviation in various grades of transitional cell carcinoma

WHO/ ISUP (2004) classification	N = 60	Mean score (nucleolar)	Std. deviation
PUNLMP	16	115.5142	46.662
Low grade	29	108.7421	31.123
High grade	15	112.2841	52.664
Total	60	112.1896	43.483

Table 5: Mean scores (extranucleolar) and standard deviation in various grades of transitional cell carcinoma

WHO/ISUP (2004) classification	N = 60	Mean score (extranucleolar)	Std. deviation
PUNLMP	16	150.5286	48.337
Low grade	29	179.4567	57.591
High grade	15	245.6734	73.823
Total	60	191.8863	59.917

Table 6: Mean scores (intranucleolar) and standard deviation in various grades of transitional cell carcinoma

WHO/ISUP (2004) classification	n = 60	Mean score (intranucleolar)	Std. deviation
PUNLMP	16	165.6285	82.631
Low grade	29	220.8742	87.345
High grade	15	261.6290	88.768
Total	601	221.8000	90.767

Table 7: Mean scores (total) and standard deviation in various grades of transitional cell carcinoma

WHO/ISUP (2004) classification	n = 60	Mean score (total)	Std. deviation
PUNLMP	16	4.5473	0.9165
Low grade	29	5.9736	1.1972
High grade	15	6.6739	1.2276
Total	60	5.7316	1.3452

Table 8: *P* values of AgNOR scores

AgNOR dots	*p* value
Nucleolar	0.932
Extranucleolar	0.021
Intranucleolar	0.081
Total	0.023

a wide age range (39 to 85 years) with a peak age incidence around 61 to 70 years.

They found that it is more commonly seen in males than females with approximate male to female ratio of 3 to 4:1,[10] where as in this study, we found that male to female ratio as 5:1.

Among the etiological factors, smokers have shown 4.02 times more risk of developing carcinoma bladder than non-smokers.[11] The number of smokers were 40.8% of the cases in this study. Smoking increases the AgNORs in the oral cavity. It has been demonstrated that larger number of AgNOR proteins are present in exfoliative cytology specimens obtained from the mucosa of the lateral tongue border of smokers who presented with no clinically visible alterations when compared to nonsmokers.[12] The average number of AgNORs/nucleus is related to the number of cigarettes per day in the mouth floor of smokers. These results suggest a possible relation between the number of cigarettes per day and an increase rate of cellular proliferation in the oral mucosal cells.[13,14]

Rehn demonstrated increased risk of bladder tumors in dye workers, most commonly associated carcinogen being aryl amine and nitro compounds.[10] We, in this study, found that occupational exposure to such chemicals were seen in 9.4% of cases. Sternberg *et al*.[10] associated vesical calculi leading to carcinoma owing to prolonged irritation of bladder mucosa. The role of ionizing radiation has also been considered as one of the etiological factors.[15] In the previous studies, hematuria and obstructive symptoms were the most common complaints in the cases of bladder carcinoma.[1,10,11] In this study, we also found similar results with 80.2% cases presenting with hematuria and 70.4% cases presenting with obstructive symptoms.

Various classifications have been used to grade carcinoma bladder amidst which WHO (1973) and WHO/ISUP (2004) are commonly used.[4,16] WHO (1973) includes Papilloma, grade I, II, and III under the classification thus limited by the drawback of labeling a low-grade lesion as carcinoma.[3] Papilloma is defined as a small usually solitary papillary lesion with a delicate fibro vascular core, which is lined by cytologically and architecturally normal urothelium without mitosis. However, the incidence of papilloma is rare.[3] We did not encounter any case of papilloma.

WHO/ISUP (2004) classification included Papilloma, PUNLMP, low grade and high-grade carcinoma.[2] PUNLMP, a new entity introduced with orderly arrangement of cells with papillae showing minimal atypia irrespective of its thickness. Incidence of PUNLMP reported is 12%. The present study had 26.6% cases of PUNLMP.[3] According to Cheng *et al*., 56% of the cases were of low grade while 32% of the cases were of high grade TCC.[17] The present study brought up 48.3% of low-grade lesions and 25.0% of high-grade lesions of TCC.

A comparison, which was done on the two classification systems, brought into consideration the fact that all cases of PUNLMP correlated with WHO grade I, which also included three low-grade tumors of the new classification. Invasion of lamina propria was seen in 75% of high grade tumors, whereas invasion of muscularis propria was seen in 62.5% of high grade cases however, one case of high grade tumor showed foci of vascular and perineural invasion. Invasion of muscularis propria is found to be associated with bad prognosis and risk of recurrence.[18] Vascular invasion has also been found to be associated with risk of recurrence.[11] Squamous metaplasia was seen in 26.6% of high grade tumors and 6.9% of low grade tumors. According to Silverberg *et al*., metaplasias are most commonly seen in high grade tumors.[1]

Several studies have been done using AgNOR staining on various lesions, which include lymphomas, cutaneous melanocytic lesions, and esophageal carcinomas.[8,19,20] The present study was done using AgNOR staining on various grades of TCC bladder. Criteria used to assess satisfactory staining of AgNOR are minimal non-specific background precipitate, fine uniformly stained AgNOR dots, and discernible AgNOR dots lying within a clustered nucleolar structure.[8]

In general, it has been observed that high-grade tumors have AgNOR dots, which are more numerous and are smaller.[21] AgNOR staining is related to cell cycle and is associated with cell proliferation.[21] The present study indicated that as the grade of the tumor increased, there was an increase in total AgNOR score along with increase in extranucleolar AgNOR score. However, some degree of overlap was seen in PUNLMP-low grade and low grade-high grade lesions. Also nucleolar and intranucleolar pattern of AgNOR dots had a variable staining pattern. According to Cairns *et al*.,[6] some degree of overlap was seen in AgNOR staining in different grades of urothelial tumors. Another study by Tomobe *et al*.[22] also showed an increase in AgNOR score with an increase in grade of tumor. However, the study by Paul Mansor *et al*.[23] showed no correlation between these two parameters. Korneyer attributed the overlap seen in AgNOR staining pattern of grade II and grade III to slight difference

in proliferation state between two grades of the tumor and to some extent to subjectivity of grading as the qualitative method with considerable intraobserver and interobserver variations.[6] According to Crocker *et al*.[21] intraobserver and interobserver variations seen in AgNOR counts ranged from 2% to 7%. This has been found to be associated with two factors; first being the tumor heterogenicity associated with variable number of histiocytes seen throughout the tumor and secondly is the section thickness. It is necessary for the section to be adequately thick to include all NOR profiles, simultaneously it should be appropriately thin so that the NOR profiles can be separated easily, thus aiding in AgNOR counts.[21]

Thus, to summarize, the two grading systems for TCC bladder, WHO/ISUP (2004) and WHO (1973), when compared, correlated well and were found to be statistically significant. Further significant correlation of the grade of the tumor was seen with the mean and extranucleolar AgNOR scores. However, some overlap was seen in between PUNLMP-low grade and low grade–high grade lesions. Also, the grade of the tumor did not correlate with intranucleolar and nucleolar AgNOR scores which showed a significant degree of overlap, thus concluding that the AgNOR scores correlated with the grade of TCC but the various staining patterns of AgNOR did not correlate with the grade of the tumor.

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