Comparison of Argyrophilic Nucleolar Organiser Regions (AgNORS) with Proliferating Cell Nuclear Antigen (PCNA) in Different Grades of Transitional Cell Carcinoma of Urinary Bladder

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Objective: To Compare Argyrophilic Nucleolar Organiser Regions (AgNORs) pattern with Proliferating nuclear antigen (PCNA) in different grades of transitional cell carcinoma.

Design: Descriptive and Comparative study.

Material and Methods: A total of fifty transurethrally resected bladder tumour samples (TUR BT) were collected from Mayo Hospital, Lahore and Services Hospital, Lahore and processed for H&E, AgNOR stain and PCNA stain. The grading of tumours were made on H&E stain. While AgNOR pattern and PCNA labeling index were recorded for each case.

Results: A significant higher proportion of cases ($p<0.02$) with 3+ AgNOR dispersion were seen in grade III tumours. The AgNOR proliferative index in grade III tumour was significantly higher ($p<0.05$) as compared with grade II tumours. The cases with AgNOR dispersion of 3+ had significantly higher mean PCNA labeling index ($p<0.02$) when compared tumours of 2+ AgNOR dispersion.

Conclusion: AgNOR and PCNA have significant role in grading of transitional cell carcinoma. The demon-stration of AgNOR dispersion is also a simple and cost effective procedure and could be considered as a substitute for PCNA labeling index.

Key words: Nuclear organizer regions, PCNA, Transitional cell carcinoma.

Malignancies of urinary bladder are one of the leading malignancies in the developed countries.[1] The incidence is also high in Northern Africa and Western Asia. The world wide estimate of carcinoma of urinary bladder of new cases is 261,000 annually.[2] Amongst these, more than 50,000 cases and estimate of 9500 deaths have been predicted in United States alone.[3] The incidence is three times more in males as compared to females and twice as common in whites as compared to blacks.[4]

Carcinoma of urinary bladder accounts for 2% of all malignant tumors and approximately 7% of all urinary tract malignancies in males. Transitional cell carcinoma constitutes the vast majority of bladder cancers.[5] It is the second most common malignancy in the genitourinary tract.[6]

Carcinoma of urinary bladder is the 8th common malignancy in Pakistan. It is the fourth common malignancy in men and its incidence is 5.4% in North West Pakistan. It has frequency of 5.75% among men and 1.61% among women.[7-9]

The argyrophilic nucleolar organizer regions (AgNORs) are loops of ribosomal DNA present onshort arms of acrocentric chromosomes which are demonstrable as black dots in the silver stained tissue section Nagatani et al[10]. AgNORs are loops of DNA in the nucleolus which code for ribosomal RNA and thus are of vital significance in the synthesis of proteins (Smith et al 1988)[11]. The proliferative activity has also been studied by AgNOR technique which has been extensively used in different malignancies.[12]

The simple AgNOR method, together with its ready application to paraffin sections, has made it a potentially interesting method for tumour biology.[13] The AgNOR staining technique has been extensively studied to find alternate technique of cell kinetic analysis other than flow cytometry.

The AgNOR staining is also used for the evaluation of both ploidy and proliferative activity of neoplasm and at the same time assess the morphology of these neoplasms.[14] While using AgNOR technique, cell imprints are superior to those of tissue section. Moreover the image analysis has shown that AgNOR area are more important than AgNOR number.[15] With increasing degree of malignancy, the number and size of the nucleoli are increased and the position of the nucleoli are shifted to the periphery of nucleus. It has been suggested that careful analysis of the number and localization on the nucleolus can be helpful in the differential diagnosis between carcinoma and atypical hyperplasia of prostate, which can be difficult in histopathological and cytopathological material.[16] Bukhari et al[17] used a modified method of AgNOR staining in brain tissue. The modified AgNOR staining was found to be simple, quick and reliable to calculate cell proliferation by detecting AgNOR size and dispersion. They recommended that AgNOR size and dispersion should be considered rather than counts only.

Immunostaing of the PCNA provides important information about cell kinetic and is performed on routinely

obtained formalin fixed paraffin embedded materials.[18] PCNA plays an essential role in nucleic acid metabolism in all eukaryotes. The PCNA protein interacts with a large number of proteins. These proteins can be divided into two groups, the first contain proteins that have a known enzymatic activity, the second contain regulatory proteins that are included in cell cycle progression.[19].The PCNA gene codes for a protein that is necessary for cellular DNA synthesis and cell cycle progression.[20].The ratio of cyclin/DNA remain constant during S phase.[21]

In tumour cells complexes, the pattern of PCNA immunoreactivity was different in papillary and primary infiltrating transition cell carcinoma.[22] The PCNA immuno-staining is useful and reproducible method of assessing one aspect of cellular proliferative activity. It has some advantages over flow cytometry in that it maintains tissue integrity and morphologic relationship.[23]

This study was carried out to compare AgNORs and PCNA Immunostaining in different grades of transitional cell carcinoma of urinary bladder.

Materials and Methods

Fifty sample of Transurethrally resected urinary bladder tumour (TUR BT) were collected in 10% formalin solution from Mayo Hospital and Services Hospital Lahore These formalin fixed tissue were processed in automatic processor for H&E staining.[24] and AgNOR staing.[25] The tissues were also stained for PCNA. The reagents of PCNA staining were purchased from DAKO Corporation, USA and used according to manufacturer's instruction.

Argyophilic Nucleolar Region Staining (Khalid et al 1996).

Following the standard procedure adopted by Khalid et al[25], Solution A was prepared by dissolving 500 mg gelatin in 25 ml deionized water along with 250 µl of formic acid (2% gelatin and 1% formic acid) and preshly prepared Solution B was prepared by dissolving different concentration of silver nitrate (1, 2, 3, 4 grams). Then the Working Solution was prepared just before the staining by mixing one volume of Solution A and two volume of Solution B. The nuclei were stained light yellow and AgNORs were visualized as brown black discrete dots of variable size within the nuclei.
Proliferating Cell Nuclear Antigen (PCNA) Staining.

The proliferating cell nuclear antigen staining was performed by Peroxidase antiperoxidase technique using commercially available reagents (DAKO Corporation USA) according to manufacturer instruction.

Microscopic Interpretation

Grading of transitional cell carcinoma was confirmed after doing H&E staining. The AgNOR count was carried out by two of the authors and mean count was calculated. Similarly variation in AgNOR size and dispersion was independently assessed by two authors according to Khan et al[26.] Any discrepancy in assessment was settled by consensus. while AgNOR proliferative index was calculated according to Mourad et al[27] as percentage of cells with more than 5 AgNOR dots.

Malignant cells were counted and average PCNA labeling index was expressed as percentage ratio of total labelled cells to the total number of cells counted.[28]

Results

The ages of the patients ranged from 43 to 72 years with a mean of 61 years. Amongst these patients 34 cases (68%) were of grade II while 16 were of grade III. The mean AgNOR count ranged from 6.25 to 12.14 with mean ±SD value of 8.65 ± 2.40 (table 1).The AgNOR dispersion of 3+ was predominant in all age groups (fig 1). A significant higher proportion of cases ($p<0.02$) with 3+ AgNOR dispersion were seen in grade III (table 2). The AgNOR proliferative index in grade III tumours was siginificantly higher ($p< 0.05$) as compared with grade II tumours (table 3). The mean AgNOR proliferative index (pAgNOR) ranged from 68 to 95% with mean ± SD of value of 82.00 ± 6.90.

Table 1: Comparison of grade of tumour with AgNOR count.

Grade of tumour	No of cases	Mean AgNOR count ± SD
II	34	8.16 ± 2.50
III	16	10.06 ± 1.39

P=NS

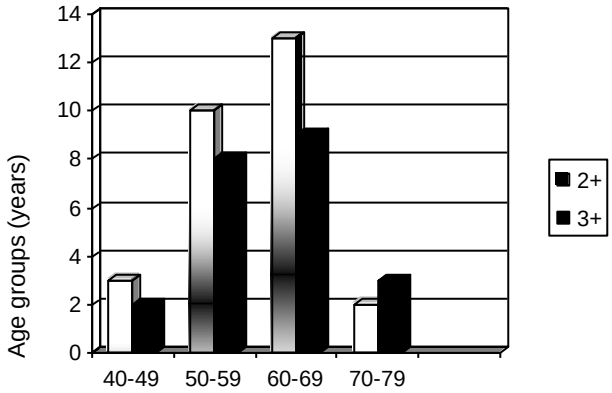

Fig. 1: *AgNOR size in different age groups in transitional carcinoma.*

The proliferating cell antigen labeling index in transitional cell carcinoma ranged from 2% to 39% with the mean ± SD value of 19.12 ± 8.10. Maximum number of cases[16] had a PCNA labeling index ranging from 11 to 20%. This is followed by 14 cases which has a PCNA labeling index of up to 10%. Mean PCNA labeling index was significantly higher in grade III when compared with tumours of grade II. The cases with AgNOR dispersion of 3+ has significantly

higher mean PCNA labeling index (p<0.02) when compared tumour of 2+ AgNOR dispersion (table 4).

Table 2: *Comparison of Grade of tumour with AgNOR dispersion.*

Grade	AgNOR dispersion		No of cases
	2+	3+	
II	15	19	34
III	1	15	16
Total	16	34	50

P< 0.02

Table 3: *Comparison of grade of transitional cell carcinoma with pAgNOR.*

Grade	No. of Cases	pAgNOR Mean ± SD
II	34	78.88 ± 4.82
III	16	88.62 ± 5.80

P< 0.05

Table 4: *Comparison of AgNOR dispersion with PCNA Labeling Index.*

Dispersion of AgNOR	No. of Cases	Mean PCNA Labeling index ± SD
2+	16	15.93 ± 13.87
3+	34	20.61 ± 13.33

p < 0.02

Discussion

Haematuria is the most common presenting complaint in patients of carcinoma of urinary bladder.[4,3] In the present study haematuria was also the commonest presenting symptoms. The mean duration of symptoms was 5 months and maximum number of cases were in 4—6 month group. Most of cases presented with grade II tumour. In our study, higher number of grade of II tumours (67.60%) presented with longer duration of symptoms as compared with grade III in tumour (56.25%). Although these finding were not statistically significant yet these are in accordance with the finding of Hendry et al.[28]

The AgNOR dispersion is another useful parameter. In our study, the AgNOR dispersion in higher tumours showed a significantly higher (p<0.02) when compared with lower grade tumours. This indicated that AgNOR dispersion is more useful parameter than AgNOR size in assessing the aggressiveness of transitional cell carcinoma.

In our study, cells of transitional cell carcinoma showed immunoreactivity for PCNA. Only five cases had PCNA labeling index of less than 10% with mean + SD value of 5.40 ± 3.22% (fig 1). The rest of cases had mean PCNA labeling index + SD 22.84±8.97 % which was statistically higher (p<0.01) than the above group. These finding are similar to that of Chen et al [29.] in which they reported mean PCNA labeling index +SD, of 12.58 ± 12.33% in superficial tumours and 34.55 ± 21.89% in invasive tumours.

All above studies suggest that proliferating cell nuclear antigen labeling index in carcinoma of urinary bladder may prove to be an objective and quantitative assay of biological aggressiveness and may provide significant prognostic information.

The PCNA labeling index had significantly higher value in high grade tumous as compared to low grade tumours.[30-,32] Similarly the PCNA labeling index in our study was significantly higher (p<0.001) in grade III tumours when compared with grade II tumours (Table 2). This pattern clearly indicates thar PCNA labeling index can be used in grading of tumours of urinary bladder. The results have shown good relation between PCNA and histopathological grade of tumour.[33] The fraction of PCNA positive nuclei ranges between 0% to 100%. In WHO grade tumour tumours, only occasional cells are positive for PCNA where as nearly all the nuclei are positive in WHO grade 3 tumours. This indicates that the cell proliferation can be assessed by PCNA/Cyclin immunostaing in transitional cell carcinoma.[34]

Finally our studies indicates that AgNOR and PCNA are reliable marker in transitional cell carcinoma of urinary bladder. The PCNA labeling index has significant role in tumour grading and future studies may prove it as reliable parameter in grading of transitional carcinoma and thus helpful in follow up of patients of transitional cell carcinoma.

However determination of PCNA labeling index is a costly and technically difficult procedure. On the other hand, dispersion of AgNOR is a simple and cost effective procedure. In view of the above mentioned facts, dispersion of AgNOR may be considered as a substitute for PCNA labeling index.

Further studies on large number of cases may be carried out to substantiate these findings.

References

1- Przybojewska B, Jagiello A, Jalmuzna P. H-RAS,K-RAS and N-RAS gene activation in human bladder cancer. Cancer Genet Cytogenet 2000; 121: 73-77.

2 Parkin DM, Pisani P, Ferlay J. Global cancer statictics. Ca Cancer J Clin 1999; 49: 33-64.

3 American Cancer Society Introduction to bladder cancer. Article online (cited 2007, July 18) available from http://American Cancer Society.com/Introduction to bladder cancer.

4 Carroll PR. Urothelial carcinoma:cancer of the bladder, ureter and renal pelvis. In:editors) Tanagho EA, McAnich J [eds]. Smith's General Urology (13th ED). San Francisco: Prentice-Hall International Inc, 1992; 341-58.

5. Metts MC, Metts JC, Milito SJ, Thomas CR Jr. Bladder cancer: A review of diagnosis and management. J Natl Med Assoc 2000; 92: 285-94.

6. Koenig F, Jung K, Schnorr D, Loening SA. Urinary markers of malignancy. Clin Chim Acta 2000; 297: 191-205.

7. Jafarey NA, Zaidi SHM. Cancer in Pakistan JAMA 1987; 178-83.

8. Khan SM, Gillani J, Nasreen S, Zia S. Cancer in North West Pakistan.Pak J Med J 1996; 35: 167-69.

9. Ahmad J, Hashmi MA, Naveed IA, Hussain A, Amin D. Spectrum of malignancies in Faisalabad 1986-1990, Pak J Pathol 1992, 3:103-10.

10. Nagatani T, Iemoto G, Miyakawa K, Ichiyama S, Takahashi Y,Uchiyama M et al. AgNOR (Nucleolar organizer region) staining in malignant melanoma. J Dermatol 1991; 18: 731-35.

11. Smith PJ, Skilbeck N, Harrison A, Crocker J. The effect of a series of fixatives on the AgNOR technique. J Pathol 1988; 155: 109-12.

12. Kodousek R, Dusek J. Demonstration of the nucleolar organizer region by silver staining (AgNOR method) in research and in histopathological practice. Acta Univ Pulacki Olumne Fac Med 1991; 131: 9-37.

13. Walker RA. The histopathological evaluation of nucleolar organizer regions proteins [Commentary]. Histopathology 1988; 12: 221-23.

14. Mourad WA, Erkman Balis B, Livingstone S, Shaukri M, Cox CE, Nicosia SV, Rowlands DT et al. Argyrophilic nucleolar organizer region in breast carcinoma. Correlation with DNA flow cytometry, Histopathology and lymphnode status. Cancer 1992; 69: 1739-44.

15. Ruschoff J, Plate KH, Bittinger A. Application of the AgNOR method to cell imprints [letter]. J Pathol 1989; 158: 333.

16. Helpap B. Observation on the number,size and localization of nucleoli in hyperplastic and neoplastic disease. Histopathology 1988; 13: 203-11.

17. Bukhari MH,Niazi s, Khan SA,Hashmi I, Perveen S, Qureshi SS,Chaudhry NA et al. Modified methods of AgNOR staining for tissue and interpretation in histopathology. Int J Exp Path 2007; 88: 47-53.

18. Morisaki Y, Shima S, Yoshizumi Y, Suziura Y, Tanaka S, Tamai S. PCNA immunostaining combined with AgNOR staining in esophageal squamous cell carcinoma to identify patients with a poor prognosis. Surg Today 1995; 25: 389-95.

19. Kelman Z, Hurwitz J. Protein PCNA interaction: A DNA scanning mechanism? Trends Biochem Sci 1998; 23: 236-38.

20. Rizzo MG, Ottavio L, Travali S, Chang CD, Kaminska B, Baserga R. The promoter of the human proliferating cell nuclear antigen. (PCNA) gene is bidirectional. Exp Cell Res 1990; 188: 286-93.

21. Bruno S, Crissman HA, Bauer KD, Darznkiewicz Z. Changes in cell nuclei during S phase progressive chromatin condensation and altered expression of the proliferation associated nuclear protein ki 67, cyclin (PCNA) p105 and p34. Exp Cell Res 1991; 196: 99-106.

22. Offer FA, Schafer G, Hittmair A,Ofer D, Grunewald K, Weyrer K et al. Proliferative activitat und expression des p-protein in udergangszellkarinomen der harnblase. [Proliferative activity and p 53 expression in transitional cell carcinoma of urinary bladder]. Verh Dtsh Ges Pathol 1993; 77: 241-46.

23. Benjamin DR. Proliferative cell nuclear antigen (PCNA) and pediatric tumour; Assaesment of proliferative activity. Pediatric Pathol 1991; 11: 507-9.

24. Stevens A. The haematoxylins. In:Bancroft JD, Stevens A (eds). Theory and practice of histological techniques. (3rd ED). London Churchill Livingstone, 1990; 107-17.

25. Khalid AW, Khan SA, Chaudrhy NA, Tayyab M, Tehseen S. Silver staining nucleolar organizer region (AgNOR) count in benign and malignant effusion. Pak Postgraduate Medical Journal 1996; 7: 3-4.

26. Khan SA,Chaudrhy NA, Khalid AW,Akhtar GN, Ibne-Rasa SN.Patterns of Argyrophilic Nucleolar Organiser Regions in Pleural and Peritoneal Effusions. JCPSP 2006; 16: 412-15.

27. Hattori K, Uchida K, Akaza H, KOISO k, Nemoto R, Harada M. Proliferating Cell nuclear antigen in human transitional cell carcinoma. Brit J Urol 1995;75: 162-66.

28. Hendry WF, Manning N, Perry NM, Whitfield HN, Wickham JEA. The effect of a haematuria service on the diagnosis of bladder cancer. In: Oliver RTD, Hendry WF, Bloom HJG (eds). Bladder cancer Principles of combination therapy (ED). London: Butterworth & Co 1981; 19-25.

29. Chen G, Lin MS, Li RS. Expression and prognostic value of proliferating cell nuclear antigen in transitional cell carcinoma of urinary bladder. Urol Res 1997; 25; 25-30.

30. Yu CC, Fletcher CD, Newman PL, Goodlad JR, Levison DA. A comparison of proliferating cell nuclear antigen (PCNA) immunostaining, nucleolar organizer region (AgNOR) staining and histological grading in

gastrointertinal stromal tumours. J Pathol 1992; 166: 147-52.

31. Yoshikawa H, lkeuchi T, lguchi H, Onodera Y, Kai Y. Expression of PCNA / Cyclin, p53, C-erb B-2 versus histlogical grade in transitional cell carcinoma of urinary baldder. J Hinyokika Kiyo 1995; 4: 253-58.
32. Iizumi T, Liyama T, Tanaka W, Okada E, Kamiyama Y, Okano Y et al. Immunohistochemical studies of proliferating cell nuclear antigen and Cathepsin D in transitional cell carcinoma of the urinary bladder. Urol Int 1997; 59: 81-87.
33. Woods AL, Hall PA, Shephard NA, Hanby AM, Waseem NH, Lane DP, Levison DA et al. The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in gastrointestinal lymphoma and its relationship to histological grade. Histopathology 1991; 19: 235-38.
34. Lipponen PK, Eskelinen MJ. Cell proliferation of transitional cell bladder cancer determined by PCNA/Cylin immunostaining. A Histopathological description. Anticaner Res 1992A; 12: 577-83.