AgNOR study in Colorectal Carcinoma with Correlation of Histopathological Grade and UICC Stage

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Abstract: *Colorectal cancer is the third most common type accounting for 10% of all cancer death. In India it is one among the common ten cancers. Dietary factors play an important role in predisposing it; only 1 to 3 % cancer is caused by Familial syndromes. Even though multiple factors play a role in predicting the outcome, in our study we use AgNOR count as prognostic indicator.* <u>Aim</u>**:** *To Study the significance of AgNOR as prognostic indicator in correlation with histopathological grade and UICC stage.* <u>Methodology</u>*: 25 random cases of operated colorectal cancer specimens were received, processed and studied using routine H&E stain and Modified AgNOR staining method. Only scattered AgNOR dots per nuclei were counted under 1000 fold magnification.* <u>Observation and result</u>*: All cases were adenocarcinoma of various grades with well, moderately and poorly differentiated shows 3.8, 3.8 and 7 dots per nuclei. Stage I, III, IV shows 3.3, 5.6 and 9.4 respectively.* <u>Conclusion</u>*: AgNOR count more than 4 per nuclei is associated with bad prognosis. AgNOR count shows significant correlation with UICC stage. Count less than 4 does not have significant correlation with tumour grade. Our study show AgNOR is a reliable prognostic indicator for colorectal cancer.*

Keywords: AgNOR- Argyrophilic nucleolar organiser region, UICC- Union of International Cancer control, DNA- deoxyribonucleic acid containing genetic code, RNA- ribonucleic acid

1. Introduction

Colorectal cancer is the third most common cancer on global basis, preceded only by carcinoma of lung and prostrate/breast in male and female respectively. Nearly 1.4 million new cases were reported in 2012, with almost 60% in developed countries [6]. In India, it is one of the common ten carcinoma with incidence rate per 100000, 4.2 & 3.9 respectively for male and female [4]. Among the Asian countries, India's incidence rate is one among the least.

It accounts for 10% of cancer death worldwide. North America, Australia, New Zealand, European countries have high incidence, in contrast to India, South America and Africa. Environmental factor, particularly dietary practices are implicated in this striking geographic contrast [7]-[15]-[17].

The factors predisposing to the higher cancer incidence are diets rich in refined carbohydrate, low fibres content, low protective micronutrients and intake of red meat. While most of cases occur sporadically, about 1 to 3 % of colorectal carcinoma occurs in patients with familial syndrome (i.e. Familial Adenomatous polyposis or Hereditary Non-polyposis colorectal cancer) or inflammatory bowel disease. The peak incidences of colorectal carcinoma occur in 6th and 7th decades. Fewer than 20 % cases occur before the age of 50 years [11].Male and female are equally affected with mean age of 62 years. About 50% of colorectal carcinoma occurs in rectosigmoid area. Shift in location towards the proximal colon occurs during the past decade [10].Right side lesions are more common with increasing age, in association with diverticular disease. The proximal lesions tend to be Polypoidal, exophytic and distal lesions tend to be annular

and encircling. Almost 98% of the cancers occurring in the large intestine are adenocarcinoma. All the colorectal cancers spread by direct extension into the adjacent structures .Metastasis occur through the lymphatics and blood vessels. The favoured sites of metastatic spread are regional lymph nodes, liver, lung and bone followed by many sites including the serosal membrane of the peritoneal cavity, brain and others.

The standard therapy for colorectal carcinoma is surgical resection. The type of surgery depends on the site of tumour. The preoperative or postoperative irradiation and chemotherapy for operable carcinoma reduces the risk of recurrence. Preoperative chemotherapy also reduces tumour size. In inoperable cases, palliative radiotherapy is shown to have good results.

Prognosis depends on the usual parameters like age, gender, location, local extension, Lymph node metastasis, vascular and perineural invasion. The proliferative index, histological type and grade, Staging, DNA ploidy, oncogenic expression and allelic loss of chromosomes guide in the prediction of outcome.

For any tumour, proliferative activity is considered to be a good prognostic parameter because it reflects aggressiveness and biological behaviour of the neoplasm. They are many methods of estimation of cell proliferation for which, flow cytometry and Ki67 monoclonal antibody estimation identify cells in all phases of cell cycle. But disadvantage of these methods are the cost and limited availability. AgNOR scoring is a simple, one step, non- expensive method which assess the proliferative activity of cells. In this study AgNOR score is used as a prognostic indicator in correlation with histopathological grading and Union of international

cancer control staging system (UICC). The prognostic value of the AgNOR scoring will assist the surgeon to decide upon appropriate treatment plan.

2. Review of Literature

Macclintock in 1934, named the area of chromosome commonly associated with nucleoli during active cell division as Nor (Nucleolar organiser region). Pasteur and bloom (1975) put forward two-step colloidal silver staining technique in controlled temperature to stain the nuclear material. Crocker and Nar in 1989 put forward single-step method using colloidal silver for staining which made argyrophilic visualisation of the nucleolar organiser region easier [3].

Nor are chromosomal segments in which RNA is encoded and it projects on the large loop of DNA. In human karotyping, the Nor is located on each of the short arms of acrocentric chromosomes 13,14,15,21, and 22 [16].At the ultrastructural level these are named as the FCS (Fibrillar centres).

Nor located by silver nitrate staining under prescribed conditions [14], are termed as AgNOR. This technique neither identifies the rRNA nor rDNA, but the acidic protein associated with sites of rRNA transcription (B23, C23, AgNOR protein and RNA polymerase-1) are identified. A quantifiable increase in the mean AgNOR count of cell population in tissue section would result if there are [18]

- Active Cell proliferation with nucleolar dissociation causing dispersion of AgNORs throughout the nucleus.
- Defect in nucleolar association resulting in AgNOR dispersion.
- High Cell ploidy resulting in increased number of AgNOR bearing chromosomes.
- Increased Transcriptional activity resulting in prominence of otherwise inconspicuous AgNORs.

In normal or benign cells, AgNORs are usually aggregated within the nucleoli, so that histopathologist will rarely be able to see more than two AgNORs per nucleus in sections. However, in malignancy the AgNORs are dispersed throughout the nucleus, enabling the counting. Thus AgNOR count in benign and malignant lesions actually denote the numerical index of dispersion, not the absolute number [5].

The argyrophilic NOR technique can be performed on paraffin section, being relatively simple and remarkably specific. Since silver salts bind to wide range of NOR associated proteins, including RNA polymerase(notably C23 protein nucleotan) and B 23 protein [8].This specificity for NORs has been confirmed by ultrastructural studies, fluorescent probe, immunoblot analysis and fluorescein tagged antibodies to NOR associated protein[2].The increased number and scattered dispersion of AgNOR is accepted as a criteria for malignancy[5].Thus, increased scattered AgNOR indicates the proliferative activity of cells , providing a potential benefit in pre-therapeutic assessment of the malignant potential.

There is a definite relationship between the grading and prognosis of cancer. Grading of colorectal carcinoma was

based on normal glandular architecture formation. Grade I (Well differentiated) is with 95% or more glandular formation, Grade2 (Moderately differentiated) is 50 – 94 %, Grade 3 (Poorly differentiated) < 50% and Grade 4 is Undifferentiated, [21].Grading(% of glands) highly variable in different studies, but it is considered as a important prognostic indicator.

The Staging is the most important prognostic indicator of any tumour. In 1937, Duke proposed staging system for rectum which classified tumour from A – C, later Kirlin &co spilt the stageA into new A(only Mucosal involvement) and B into B1 & B2[19].Astler and Coller, in 1954 modified it by splitting stage C into C1&C2.Kyriaskos later added stage D for distant Metastasis[20].

In our study, UICC staging with changes proposed in 7th edition was followed [4]-[22].

3. Aims and objectives

This study is undertaken to enumerate the average number of AgNOR dots per nucleus in colorectal carcinoma and to study its prognostic significance. It also studies the correlation with histopathological grade and UICC stage.

4. Material and Method

The 25 randomly selected cases of colorectal carcinoma operated for radical purpose in Govt. Vellore Medical College, Department of Surgery in 2014-2016 were studied. Specimens were received in the Department of Pathology with complete clinical data. Cases with preoperative adjuvant chemo/radio therapy and those with incomplete data were excluded from the study. Specimens were fixed in buffered formalin and routinely processed. After routine H & E staining, microscopic examination (400fold magnification) was done. Subsequently AgNOR staining by modified procedure of Crocker was done [2] using working solution composed of two part 50 % silver nitrate and one part gelatine solution (prepared immediately before staining).The stain layered slides were placed in a dark room for 40 minutes at room temperature, later washed in distilled water, dehydrated, cleared and mounted in DPX. A minimum 100 cells were examined and AgNOR dots per nucleus were counted under 1000 fold magnification.

Only scattered AgNOR dots were counted, as it is largely independent of staining time variation and have proved to be objective, reliable and reproducible prognostic factor. Whereas clustered AgNOR varies depending on stain intensity and staining time. So in our study only mean scattered AgNOR per nucleus were considered. Normal colonic mucosa shows mean AgNOR 1-3 per nucleus of which none is scattered. In colorectal carcinoma, the mean AgNOR count 5-9 per nucleus of which scattered AgNOR dots are 2-6 per nucleus. Red cells with no AgNOR or lymphocytes with single central AgNOR are used as internal control.

After examination for mean scattered AgNOR count, it was correlated with following available data such as

histolopathological type, grade and UICC Stage using TNM classification.

5. Observation

Most of the cases were in age group 41- 60 years, of which 21 were male. Of the 21 male, 17 has tumour in rectosigmoid area, 3 in ascending colon and 1 in descending colon. In all the female patients, tumour arises from rectosigmoid area. In all the cases, Histopathological type was adenocarcinoma of different grades.

Out of 25 cases, 16 are well differentiated tumour, with scattered AgNOR count varying from 3.1 to 6.3 per nucleus with Mean value 3.8. Four cases were moderately differentiated, with scattered AgNOR count varying from 3 to 5.5 per nucleus, with Mean value 3.8. Five cases were poorly differentiated, with scattered AgNOR count varying from 5.2 to 12.5 with Mean value 7.

Figure 1

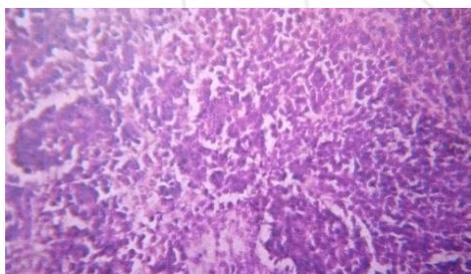Picture showing Hemicolectomy specimen (ascending colon) with growth

Figure 2

Poorly differentiated adenocarcinoma
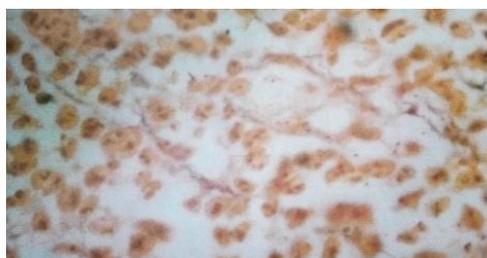
Under 100x (H&E staining)

Figure 3

Poorly differentiated adenocarcinoma under 1000x (colloidal Silver staining) showing 5-6 dots per nucleus.

Table 1: Tumour grading with AgNOR count

S.no	Grade	No. of case	Mean AgNOR count
1	1	16	3.8
2	2	4	3.8
3	3	5	7

As per TNM classification 3,14,6 and 2 cases occur in T1, T2, T3, T4 stages respectively. Mean scattered AgNOR count are T1-2.9, T2-3.7, T3-5.7 and T4 -9.4.

Table 2: Tumor (T) stage with AgNOR count

S.no	Tumor (T)	No. of cases	Mean AgNOR count
1	T1	3	2.9
2	T2	14	3.7
3	T3	6	5.7
4	T4b	2	9.4

15 Cases with no Nodal metastasis have Mean scattered AgNOR count is 3.3 per nucleus. Six N I cases have Mean scattered AgNORs per nucleus is 5.6, N2a and N2b with 2 cases each show Mean count of 5.8 and 9.4 respectively.

Table 3: Nodal (N) stage with AgNOR count

S.no	Node (N)	No. of cases	Mean AgNOR count
1	N0	15	3.3
2	N1	6	5.6
3	N2a	2	5.8
4	N2b	2	9.4

Two cases with distant metastasis show Mean count 6.3 and 12.5 respectively. As per UICC staging, 15 cases of stage I show Mean count of 3.3, 8 cases of stage III show Mean count of 5.6 and 2 cases of stage IV show mean count 9.4. No Stage II case was present in our study.

Table 4: UICC stage with AgNOR count

S.no	Stage	No. of cases	Mean AgNOR count
1	I	15	3.3
2	III(a,b,c)	8(2+5+1)	5.6
3	IV(a,b)	2(1+1)	9.4

6. Discussion

Mean scattered AgNOR count less than 4 per nucleus carries good prognosis compared to count more than 4 per nucleus [13]. Another study on renal cell carcinoma demonstrated that low count had nearly 100% 5 year survival rate compared with high count having significant evidence of metastasis and mortality [1].

AgNOR had been proven as a major and independent prognostic factor in the cases of colorectal adenocarcinoma treated exclusively by surgery [12]-[9].

In our study, both well and moderately differentiated tumour with Mean count less than 4 (3.8) have good prognosis compared to poorly differentiated tumour with Mean count 7.

The difference in survival between well, moderate and poorly differentiated tumours was significant in the group

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with high AgNOR count [23]. Our study shows no significant difference when the count is < 4.

Similar Study shows 5 year survival rate of Stage I, II, III and IV as 97%, 87%, 73% and 22% respectively [24].

Stage I cases in our study with Mean AgNOR count < 4 carries very good prognosis compared to Stage III and IV with Mean AgNOR 5.6 and 9.4 respectively. Among the two stage IV cases, one case with well differentiation & Mean count 6.3 had metastasis to bladder. The other case with poor differentiation & Mean count 12.5 had metastasis to bone, bladder and peritoneum.

Cases with nodal metastasis (NI & N2) with > 4 dots per nucleus had bad prognosis compared to No cases. The result of this study demonstrates a significant correlation between Mean Scattered AgNOR score and UICC (TNM) staging. In cases with high Mean AgNOR count, correlation with grading was noted.

7. Conclusion

From this study, it is concluded that <4 scattered AgNOR per nucleus carries good clinical outcome regardless of their grade. Count >4 per nucleus carries bad prognosis. AgNOR count correlate well with UICC staging system (TNM).AgNOR count is a reliable prognostic predictor for colorectal carcinoma.

8. Future Scope

Similar studies in future, across institutions with larger sample size and longer surveillance period will shed more light in the area of clinical behaviour of colorectal carcinoma. That data will assist the clinician in planning tailor-made treatment for the colorectal carcinoma patients.

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