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Nucleolar organizer regions (AgNORs) as a proliferative index in benign, premalignant and malignant colorectal lesions

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Abstract
Aims and Objectives: To establish the role of AgNORs in differentiating the benign from the pre-malignant and the malignant lesions of the colorectal. To establish the degree / grade of malignancy according to the AgNOR count.
Material and methods: A retrospective study was conducted on 35 cases from July 2004 – July 2006 on the biopsies which were obtained from colorectal lesions. Two slides were prepared for each case – one was stained with the haematoxylin and eosin stain and the other was subjected to silver staining. The data was analyzed by using the independent t-test and ANOVA for the intergroup comparisons.
Result: The results showed that the mean AgNOR count of the carcinomas was significantly higher than that of the benign tumours and inflammatory lesions ($p < 0.05$). The AgNOR dots tend to be small, homogenously stained and regular in the benign lesions but the dots became irregular, large dots or bizarre clusters in carcinoma and adenoma with dysplasia.
Conclusions: AgNOR staining can be considered as a useful addition to diagnostic pathology. This study was helpful in the detection of the importance of AgNORs in differentiating the benign, pre-malignant and malignant lesions of the colorectum and it could be considered as an important tool along with the histopathological criteria for the evaluation of the proliferative activity of the cell.

Keywords: AgNORs, colorectal lesions, carcinoma, adenomas, silver staining, ulcerative colitis, crohn's disease

Introduction

Ulcerative colitis is an ulcero-inflammatory disease limited to the colon and affecting only the mucosa and sub-mucosa except in most severe cases [1]. Ulcerative colitis is associated with polyarthritis, uveites ankylosing spondylitis, hepatic involvement and skin lesions [2]. Its incidence in the United States and UK is about 4 to 6 per 100,000. It is common in female and more in white than blacks [1].

Crohn's disease is an inflammatory bowel disease, sharply delineated bowel segment might be affected with intervening unaffected skip areas. Crohn's disease is characterized by the presence of non-caseating granuloma, fissures and fistula. It is also has systemic manifestation [3]. It is common in western countries, its incidence 1 to 3 per 100,000 [4].

Adenomatous polyps are neoplasm that range from small pedunculated lesions to large neoplasms that are usually sessile. The prevalence of colonic adenomas is about 20% to 30% before age of 40 but its prevalence rises to 40% to 50% after age of 60. Adenomatous polyps are divided into three subtypes [4, 5].

- Tubular adenomas: small polyp commonly found in colon. It is formed of stalk that composed of fibro-muscular tissue and prominent blood vessels with normal mucosa [4].
- Villous adenoma: cauliflower like villous projections covered by dysplastic columnar epithelium. Most commonly found in rectum and recto-sigmoid colon [4].
- Tubulo-villous adenomas: a mixture of tubular glands and villous projections may harbour severe dysplasia (carcinoma in situ) [4].

Carcinoma of colon and rectum is a disease of western wolf life style [4]. It is by far the most common and most curable carcinoma of gastrointestinal tract [6, 7]. The aetiology of colorectal carcinoma may be multifactorial, such as diet factors and gene mutation. Some cases of colorectal carcinoma are associated with familial polyposis syndrome. Few cases of colorectal carcinoma occur as a complication of pelvic irradiation [8]. Microscopically the lesion may range from well differentiated to poorly differentiated carcinoma. Staging of colorectal cancer arrange from grade I with no metastasis no regional lymph node involvement to stage V with distance metastasis [9, 10].

Some of the benign colorectal lesions such as adenomas have the potential to become malignant. The malignant risk within an Adenomatous polyp is correlated with polyp size, histological features and severity of epithelial dysplasia [11, 12]. Compared to small tubular adenoma, large villous adenoma carries high risk of malignancy and compared to adenoma with mild epithelial dysplasia, the ones with moderate to severe dysplasia carries higher risk of malignancy [10]. Ulcerative colitis also considered as a premalignant lesion therefore, the detection of ability of a lesion to harbour malignant potentiality is of a big clinical importance.

Nucleolar organizer regions (NORs) are loops of ribosomal DNA which occur in the nucleoli of the cells on the short arms of the acrocentric chromosomes [13, 14, 15, 16, 17, 18, 19]. The interphasic NORS can be clearly visualized at the light microscopical level by using a silver reaction which stains the acidic proteins of the NORS on routinely prepared histopathological and cytological samples [20]. Since, the routine histopathological techniques do not demonstrate all the

features which can be used to differentiate the malignant lesions from the benign ones and as many studies showed the correlation between the changes in nucleolar features and the tumour pathology [21], NORs method has been used to distinguish between benign and malignant tumours [22]. Using a simple Argyrophil technique AgNORs [23], NORs can be seen in the cytoplasm as black dots throughout the nucleolar area. In quantitative terms, the number of AgNORs per nucleus suggests it to be a marker of the proliferative activity of the cell. It has been found that highly malignant neoplasm have AgNORs which are more numerous as and smaller than those which are benign or less malignant [24]. AgNORs are aggregated to form a solitary, rounded structure [25].

Material and Method

92 cases of colorectal lesions were taken from the files of the pathology department of Benghazi University at the period between 2007–2009. The cases was selected as following: 38 case of colorectal carcinoma, 29 cases of colorectal adenomas, 14 cases of Crohn's disease and 11 cases of ulcerative colitis. The blocks of the selected cases were utilized as a material of the study. The age of the patient, sex and the presentation were recorded. Each block underwent cutting into 2 sections 3-5 µm thickness, one section for Hematoxylin and Eosin (H&E) staining to confirm the diagnosis. Another section for Argyrophil silver stain for (AgNORs) Nucleolar organizer regions. Results from the above 92 cases were collected and tabulated.

Technique for the Argyrophil stain [26, 27, 28]

The AgNORs technique consists of incubation of the de-waxed, hydrated sections with a mixture of 2g / dl gelatine in 1g/ dl formic acid and 50 g/dl aqueous silver nitrate (in the proportion of 1:2) under dark light condition for 40 minutes, then washed with de-ionized water, not counter, stained, dehydrated, cleared and mounted in synthetic resin. Under oil immersion, objective two hundred nuclei of the lesion cells were counted randomly in each case for the presence of clearly defined black dots after careful focussing. The average number of silver stained dots per nucleus was calculated as mean number of AgNORs, (mean AgNORs) and the percentage of nuclei exhibiting five or more AgNORs per nucleus was recorded as proliferative index of AgNORs (p AgNORs) [14]. The results which were obtained in the counting procedure were analyzed statistically by using the Student's t-test and one way analysis of variance (ANOVA) for intergroup comparisons [29, 30, 31].

Results

In this study the age of the patients ranged from 18 to 80 years with a mean age of 50.3 $\pm$ 4.56 years (median= 50 year). Males found to be more affected with carcinoma (66.7%), adenoma (80%) and Crohn's disease, whereas ulcerative colitis showed an equal percentage for both male and female (50%) (data not shown). The percentage of the histopathological varieties of colorectal lesions was as following: 41.3% for carcinoma, 31.5% for adenomas and 15.2%, 11.9% for Crohn's disease and ulcerative colitis respectively (figure 1). AgNORs were visible as black dots within the nuclei of the epithelial cells.

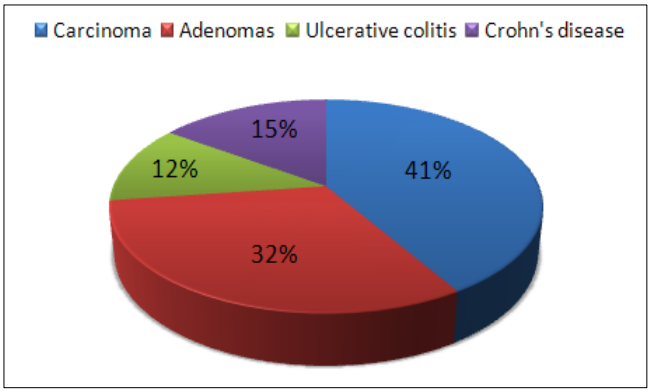

Fig 1: The percentage of histopathological varieties of colorectal lesions.

Pooled AgNORs mean number in Crohn's disease was 1.22 $\pm$ 2.18 with 0% while pooled AgNORs mean number in ulcerative colitis was 3.02 $\pm$ 2.63 with a percentage of 8.75%. For adenomas, pooled AgNORs mean number of 2.84 $\pm$ 1.87 with 11.6%, pooled AgNORs mean number of 4.52 $\pm$ 3.44 with 46.7% and pooled AgNORs mean number of 6.14 $\pm$ 5.14 and 82.3% for adenoma with mild, moderate and severe atypia respectively. For carcinoma, the pooled mean of AgNORs was 6.90 $\pm$ 1.23 and the pooled percentage of AgNORs was 78.1%. The AgNORs mean number (mAgNORs) and percentage (of nuclei exhibiting more than 5 dots) (pAgNORs) in the nuclei of colorectal lesions are shown in table 1.

Table 1: The AgNORs mean number and percentage in the nuclei of different colorectal lesions.

Type of lesion	m-AgNORs	p-AgNORs
Carcinoma	6.90 $\pm$ 1.23	78.1%
Adenoma with severe dysplasia	6.14 $\pm$ 5.14	82.3%
Adenoma with moderate dysplasia	4.52 $\pm$ 3.44	46.75%
Adenoma with mild dysplasia	2.84 $\pm$ 1.87	11.6%
Ulcerative colitis	3.02 $\pm$ 2.63	8.75%
Crohn's' disease	1.22 $\pm$ 2.18	0%

Although there was no significant difference between mAgNORs in carcinoma and mAgNORs in adenoma with severe dysplasia ($P > 0.05$) (figure 2), mAgNORs in carcinoma was significantly higher than that in adenomas with mild and moderate dysplasia ($P < 0.0001$) (figure 2). mAgNORs in carcinoma and adenoma with severe dysplasia were also significantly higher than that in ulcerative colitis and Crohn's disease ($P < 0.0001$) (figure 3A & B). Furthermore, mAgNORs in adenoma with moderate dysplasia was significantly different from mAgNORs in Crohn's disease ($P < 0.0001$) and, to a less degree from mAgNORs in ulcerative colitis ($P < 0.001$) (figure 3C). Interestingly, mAgNORs in adenoma with mild dysplasia was significantly different from mAgNORs in Crohn's disease ($P < 0.001$) but the difference between mAgNORs in adenoma with mild dysplasia and mAgNORs in ulcerative colitis was insignificant ($P < 0.05$) (figure 3D).

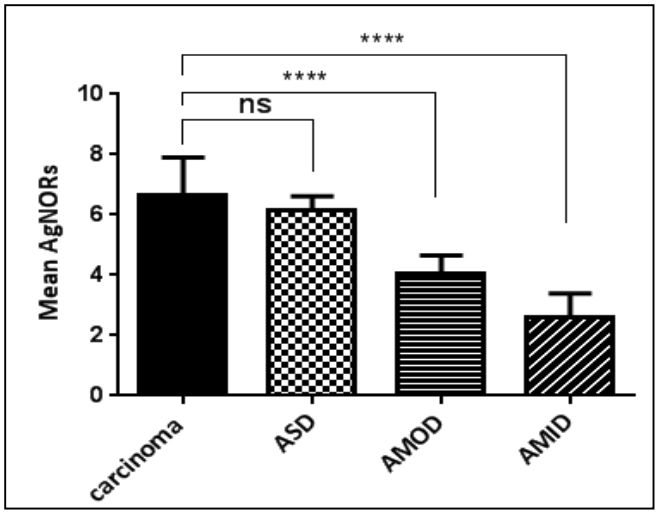

Fig 2: Mean AgNORs showed direct proportion with carcinoma and dysplasia. Carcinoma shows the highest mean number of AgNORs followed by ASD, AMOD and AMID. No significant difference between mean AgNORs in carcinoma and mean AgNORs in ASD but the difference was significant when carcinoma is compared to AMOD and AMID. ASD = adenoma with severe dysplasia, AMOD = adenoma with moderate dysplasia and MID = adenoma with mild dysplasia. ns = not significant, ****P< 0.0001. 200 nuclei were counted for each case.

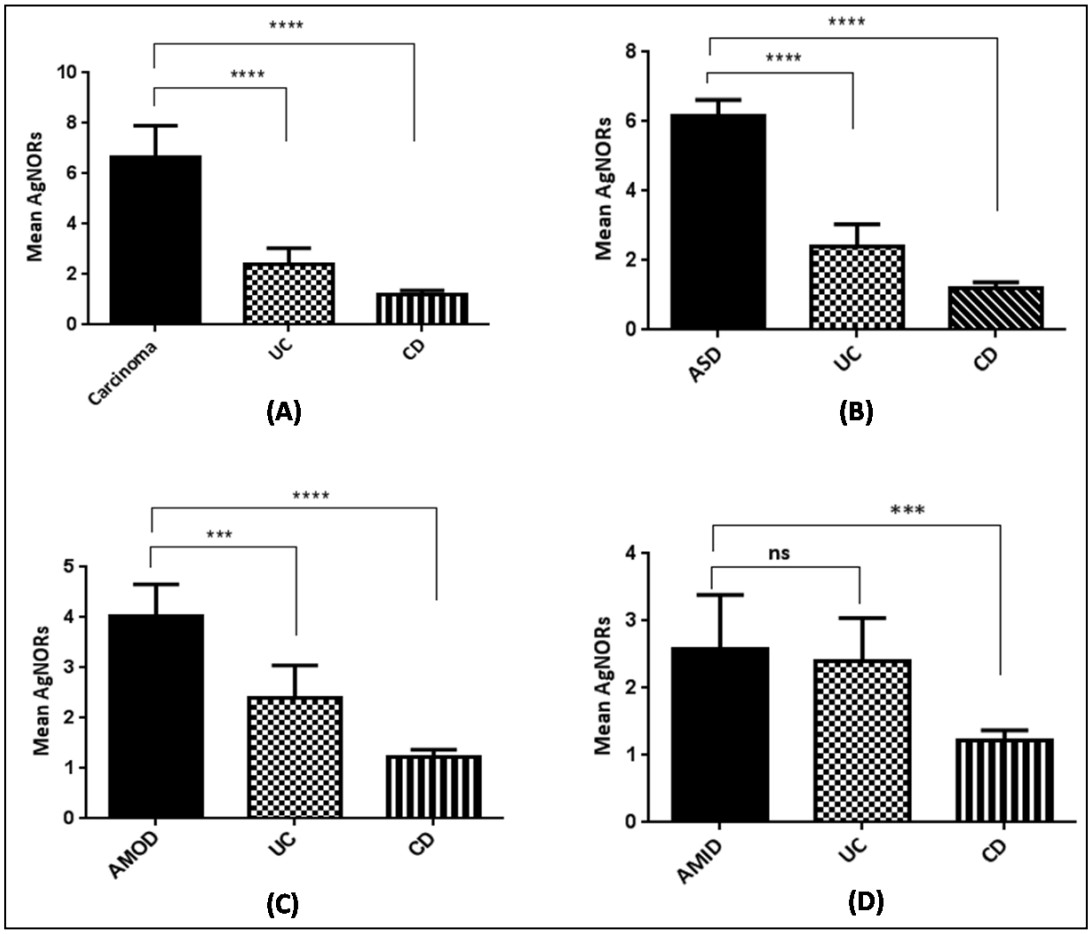

Fig 3: Compared to carcinoma and adenoma, inflammatory bowel diseases showed low mAgNORs. (A & B) mAgNORs in carcinoma and ASD were significantly higher than that in ulcerative colitis and Crohn's disease. (C) Mean AgNORs in AMOD significantly higher than that in CD and to a less degree higher than mAgNORs in UC. (D) Mean AgNORs in AMID did significantly differs from mAgNORs in UC but it is significantly higher than mAgNORs in CD. UC =ulcerative colitis, CD =Crohn's disease, ASD =adenoma with severe dysplasia, AMOD =adenoma with moderate dysplasia and AMID =adenoma with mild dysplasia. ns= not significant. ***P< 0.001, ****P< 0.0001. 200 nuclei were counted for each case.

A representative photos of the H & E stain and the AgNORs stains are shown in figures 4, 5, and 6. The AgNORs dots in malignant and pre-malignant lesions looked irregular clumped and dense (figure 6.A, B and C). Conversely, inflammatory lesions showed small regular dots (figure 6.D and E)

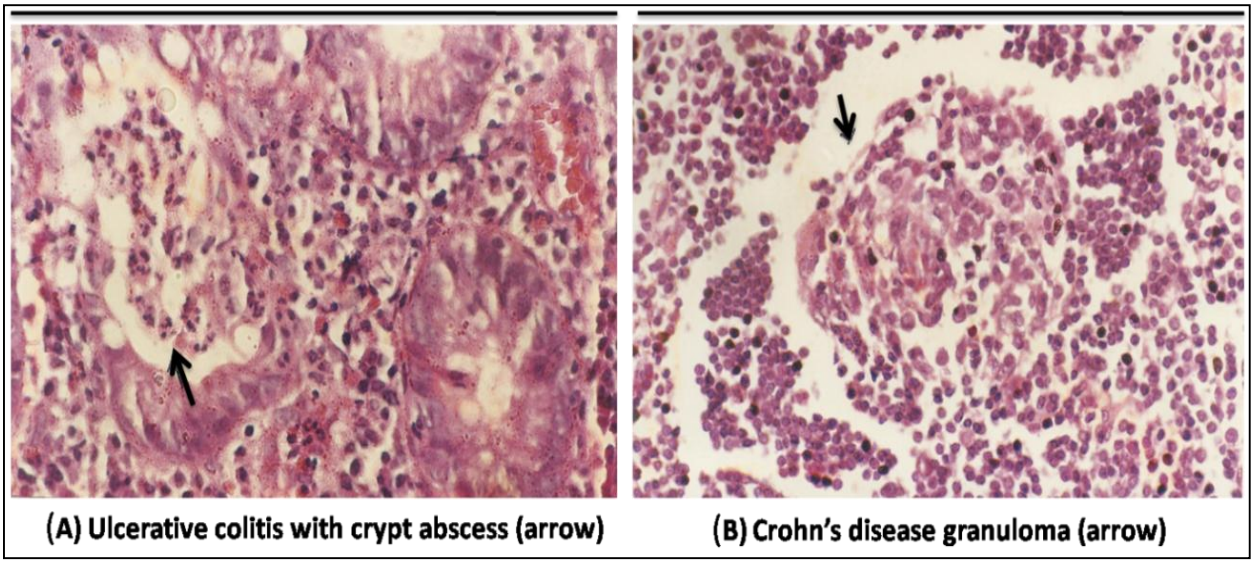

Fig 4: Inflammatory bowel diseases. Crohn's diseases and Ulcerative colitis are benign colorectal lesions. (A) Crypt abscess (arrow) seen in ulcerative colitis. (B) Non-caseating granuloma (arrow) which is seen in Crohn's disease. H & E stain. X 400.

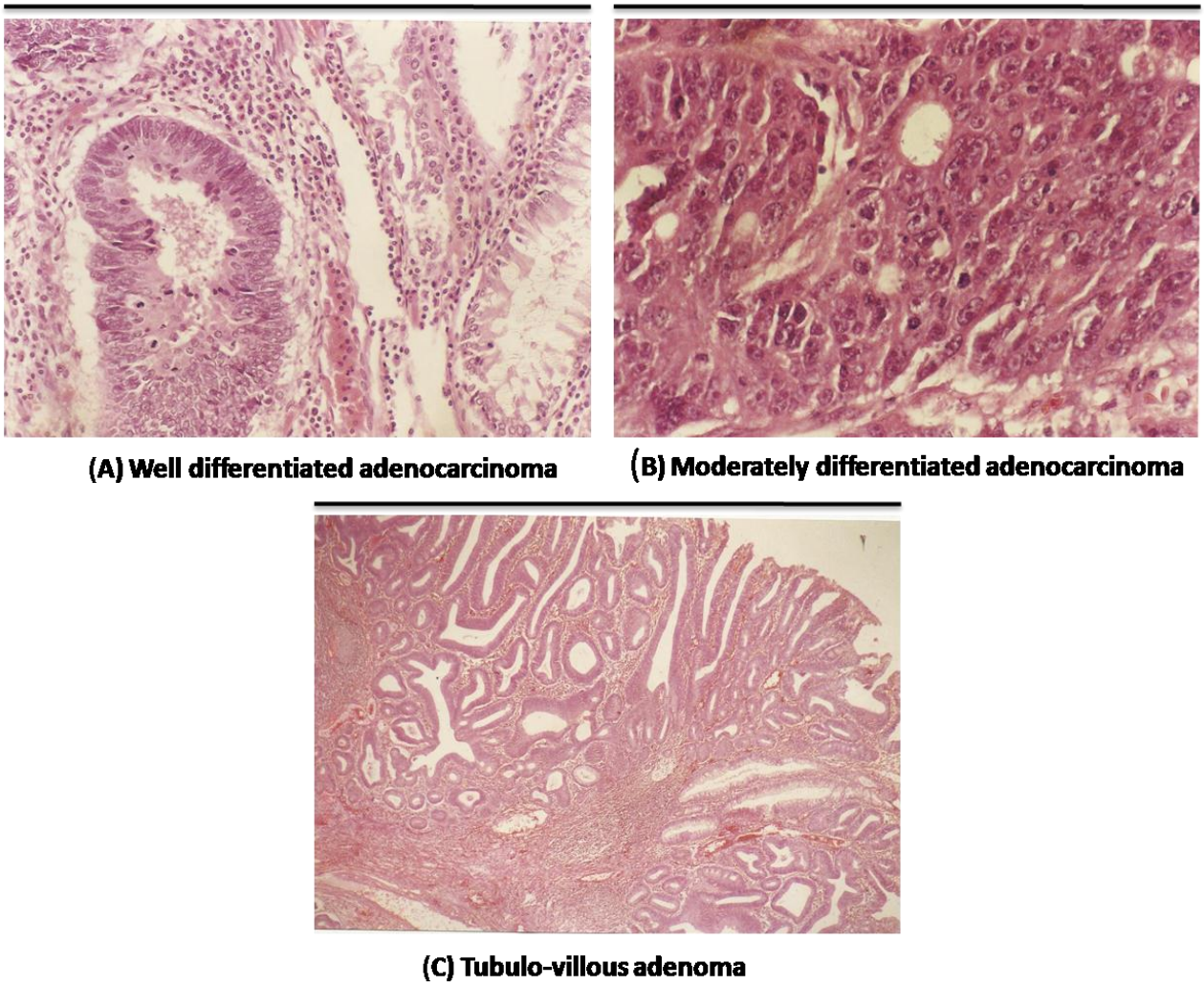

Fig 5: Malignant and pre-malignant colorectal lesions. (A) Well differentiated adenocarcinoma, the glandular appearance is maintained. (B) Moderately differentiated adenocarcinoma, show diffuse sheet of malignant cells and residual glands. (C) Tubulo-villous adenoma with cauliflower pattern. H & E stain. X 200.

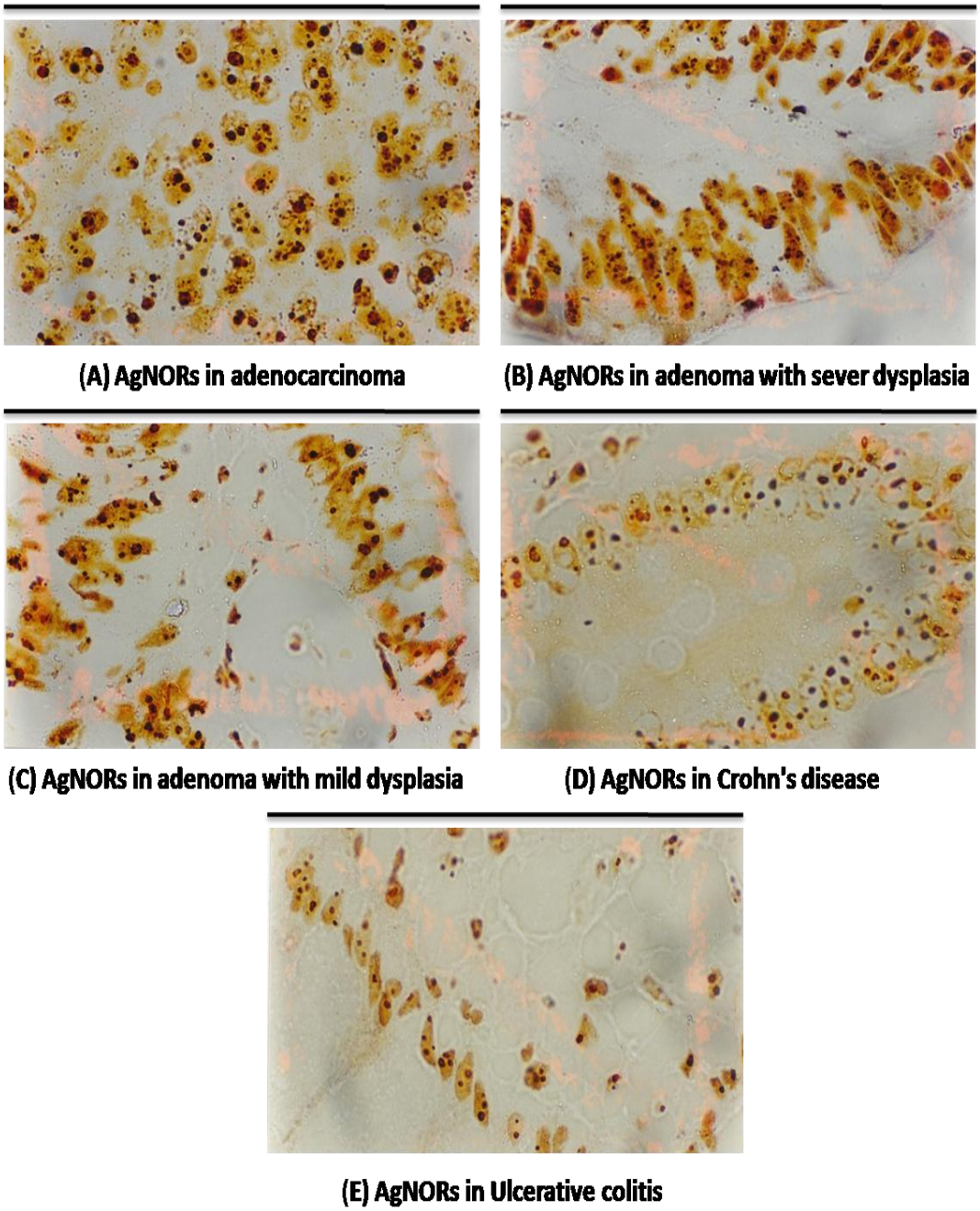

Fig 6: AgNORs in different colorectal lesions. (A & B) AgNORs in Carcinoma and adenoma with severe dysplasia were abundant with irregular sizes and shapes. (C) AgNORs in adenomas with mild dysplasia is less than that in carcinoma and adenoma with severe dysplasia but more than the amount of AgNORs in inflammatory bowel diseases (D & E). AgNORs in Crohn's disease and ulcerative colitis are small and regular spots. The AgNORs mean number is directly related to the severity of the dysplasia. X 1000.

Discussion

Diagnosis of small superficial colorectal tumours as adenomas and carcinomas has become a problem due to conflicting views regarding colonic carcinogenesis; whether the carcino-genesis follows an adenoma-carcinoma sequence or a de novo cancer is unclear [32]. The present study was performed to assess the importance of AgNORs in differentiating benign, premalignant and malignant lesions of colorectum. The malignant cells usually show a rise in the synthesis of normal and abnormal products and thus show a significant rise in the AgNORs material [33, 34].

In our study, it was observed that the AgNORs dots tended to be small, homogenous stained and regular in the nucleus of benign lesions, whereas significant irregular big bizarre clusters were seen in colorectal carcinoma and lesions with sever dysplasia. In a study by Jain *et al.* 1997 the AgNORs mean number of nuclear organizer (NORs) per nucleus ranged between 3.59 and 6.70 in colorectal carcinoma [35].

In our study, the mean number of NORs per nucleus was slightly higher (5.6 and 8.23) and this can be explained by the differentiation of the tumour as the carcinoma in our study may be less differentiated compared to Jain *et al.* study. In addition, compared to Jain *et al.* 1997 study, our study show approximately similar statistics of the mean number of NORs per nucleus (3.00-4.00) for different types of adenomas.

In our study, there was no statistical significant difference between AgNORs counts in invasive carcinoma and adenoma with severe dysplasia. These results was similar to that

described by Shimada and Suzuki, 1998 [36]. The similarity between carcinoma and severe dysplasia is related to the behaviour of the severe dysplasia which is considered as carcinoma in many cases.

Regarding inflammatory bowel diseases, for ulcerative colitis, we detected a higher mean number of AgNORs (3, 02) compared to Jain *et al.* 1997 (2.75), but same result (1.24) was detected for Crohn's disease in both study [34].

In a study by Muscara *et al.* 1997 [37], there was no difference between AgNORs counts in carcinoma and ulcerative colitis with sever dysplasia. In our study the ulcerative colitis cases did not show severe type of dysplasia, only mild degree of dysplasia was detected in ulcerative colitis, therefore, there was a statistical difference between the mean number of AgNORs in carcinoma and ulcerative colitis. Similar to Muscara study we detected no difference between carcinoma and adenoma with severe dysplasia. So based on the results of both studies, there was no differences in the mean number of AgNORs in carcinoma and severe dysplasia in general, i.e. severe dysplasia is associated with increased number of AgNORs, no matter what the type of the primary lesion. Interestingly, our study showed no difference between AgNORs in adenoma with mild dysplasia and ulcerative colitis, but AgNORs mean number in Crohn's disease was significantly differ from that in mild dysplasia, this can be explained by the features of ulcerative colitis as premalignant disease that carry the risk of progressing into carcinoma. This may indicates that AgNORs is a sensitive test to detect the ability of the lesion to progress into malignancy. In our study, the mean number of AgNORs in adenoma with moderate dysplasia was significant compared to Crohn's disease ($P< 0.0001$) but the difference was less when ulcerative colitis was compared to adenoma with moderate dysplasia ($P< 0.001$) which is another evidence to support the sensitivity of the AgNORs stain to detected ability of benign lesion to change into malignancy.

However, there are certain limitations in our study like the resolution of individual AgNORs within relatively small nucleolus and the affinity of the nucleolus for silver stain which obscures the individual AgNORs in cases of intense staining.

Conclusion

The main objectives of this study were to study the proliferative activity of different types of colorectal lesion, which are carcinoma, adenoma and inflammatory bowel disease (Ulcerative colitis and Crohn's disease) with the help of nucleolar organizer regions (AgNORs) as proliferative index, in order to study the fate and prognosis of these lesions. The analysis of the results of these study showed that the use of the AgNORs stain is a highly accurate method to detect the lesions with high proliferation rate and the malignant tendency of the lesion can be predicted. Large number of cases, must be study for more accurate results. The outcome of the patient and the result of treatment must be included in the study. Also *P53* tumor suppressor gene expression most be study for early diagnosis and as prognostic factor.

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