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RESEARCH ARTICLE

Assessment of Morphometric analysis, AgNOR Score & IHC expression of Ki67 in Gallbladder carcinoma.

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Abstract

Gallbladder carcinoma is rare but is a highly lethal neoplasm. The present study evaluates the expression of AgNOR parameters, morphometry and Ki67 index by IHC in neoplastic gall bladder lesions according to histological grade. Tissue sections from 50 cases of gallbladder disease, including 25 cases of gallbladder carcinoma and 25 cases of chronic cholecystitis were studied. On morphometry, mean nuclear area and N/C ratio of Neoplastic group was significantly higher as compared to that of Non-neoplastic group. Ki-67 expression was studied in neoplastic cases only where it ranged from 15 to 40%. Mean Ki67 expression was 28.20±7.83%. AgNOR count ranged from 1 to 14. In neoplastic cases mean AgNOR count was 7.88±2.42 as compared to 2.32±1.07 in non-neoplastic cases, thus showing a significant difference between two groups. On receiver operator curve analysis, a cut-off value of Nuclear area $\geq$86 was observed to be 96% sensitive and 84% specific in detection of neoplastic cases. A cut-off value $\geq$0.433 for N/C ratio was observed to be 100% sensitive and 84% specific in diagnosis of non-neoplastic condition. An AgNOR cut-off value $\geq$5%, was 100% sensitive and 100% specific in discriminating between neoplastic and non-neoplastic lesions. In neoplastic group, N/C ratio, AgNOR & Ki-67 expression did not show a significant association with histopathological type but showed a significant association with histopathological grade.

On the basis of above evaluation, it can be concluded that AgNOR count, Ki67 and cytomorphometric parameters i.e. N/C ratio show a high discriminant value in discrimination between neoplastic and non-neoplastic lesions. These markers also showed a significant association with the histopathological grade, however, owing to fewer numbers of cases, the association between histopathological type and these markers remains unsubstantiated.

Introduction:-

Carcinoma gallbladder is one of the most common malignancies of the gastrointestinal tract. Its incidence is quite high in northern India, especially along the gangetic belt (Dhir V et al., 1999). In India, cancer of GB shows varying geographic distribution, as the incidence is much higher in North Indian population as compared to South India (NCRP, 2001) Cholelithiasis, especially untreated chronic symptomatic gallstones, is one of the main risk factors of gallbladder cancer (Chen A et al, 1991 & Singletary BK et al.,1986). The prognosis of gallbladder carcinoma is poor and less than 5% of the patients are still alive five years postoperatively. There is paucity of knowledge on the proliferative features of normal or chronically inflamed gallbladder and the mechanisms of development of GB

cancer (Roa EI et al., 2009). Most of the gall bladder cancer cases have regional disease or distant metastases at presentation. Therefore, the prognosis in gallbladder disease is poor, with 5-year survival rates of 15-20% (Schottenfeld et al., 2006) Considering the high rate of mortality attributable mainly to late detection of disease at an advanced stage, early diagnosis remains to be one of the most important determinants of the outcome.

NORs are segment of DNA coding ribosomal genes and are situated on the short arms of acrocentric chromosomes (Bernardi et al.,2006).These specific portions of DNA, called rDNA that, by the enzyme RNA-polymerase-1, code for the transcription of ribosomal RNA (rRNA). This rRNA inside the ribosomes is responsible for protein synthesis of the cell. Protein synthesis is a necessary step in the process of proliferation (Bukhari et al.,2007) These NOR represent loops of DNA that are associated with specific proteins including RNA polymerase **IB23** protein, and **C23** protein (Ahsan et al.1990, Anselmi et al., 1990). These proteins can be easily demonstrated by means of argyrophilic techniques (Siddiqui et al., 1999). The silver staining method for argyrophilic NOR-associated proteins (AgNORs) has been used to visualize NORs in chromosomes (Goodpasture et al., 1975) and nucleoli (Ploton et al., 1984). The number and size of AgNORs correlate with the level of rDNA transcription (Hofgartner et al., 1979, Morton et al., 1984) , the degree of cell proliferation, evidenced by the percentage of cells in S-phase (Field et al., 1984), and the growth fraction determined by Ki-67 monoclonal antibody (Macartney et al., 1988). Their appearance of nucleoli may be useful in determining whether a cell is in interphase, or late, or early phases of cellular division (Raymond et al., 1989) Application of AgNOR staining to conventionally fixed and processed, paraffin sections has made this technique a useful tool in diagnosis of human malignancies (Anastassova et al., 1977). Estimation of AgNORs parameters (number, size and distribution) has been applied in tumor pathology both for diagnostic and prognostic purposes. AgNOR number and distribution in the nucleus (configuration) were useful in the detection and prognosis of some neoplasia such as renal, bladder, and pharyngeal carcinoma, multiple myeloma, and skin melanocyte lesions (Shahida et al., 2006).

Image analysis is one of such quantitative techniques which deal with morphometry, object counting and cytometry. Originally, it was used to describe the extraction of numerical information from pictures. Presently, the software techniques for image analysis have improved markedly (Khanduri et al., 2003). Histopathology, morphometry along with various ancilliary techniques such as IHC, is very much helpful in determining the prognosis of tumor. Morphometry is the process of measuring cellular and tissue architectural feature with regard to size (area, volume), shape, number of objects and distance (Weibel et al., 1979) Morphometric techniques have been used to analyze the discriminative capability of various parameters, especially cytological and nuclear feature. Cell image analysis aims to determine numeric values for the purpose of cell characterization.

The Ki-67 protein is a cellular marker for proliferation (Scholzen et al., 2000). It is strictly associated with cell proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, AND MITOSIS), but is absent from resting cells (GO).Ki-67 is an excellent marker to determine the growth fraction of a given cell population. The fraction of Ki-67 positive tumor cell (the Ki-67 labeling index) is often correlated with the clinical course of cancer. Original Ki-67 antibody (and the reason why it has essentially supplanted the original antibody for the clinical use) is that it can be used on formalin-fixed paraffin-embedded sections, after heat-mediated antigen retrieval.

Despite the availability of generalized research on prognostic role of AgNOR, Ki-67 and in addition to morphometry, their applied use in prognosis of gall bladder carcinoma is limited. Moreover, there is no single study showing the use of simultaneous above parameters on gall bladder cancer to access a better prognosis, hence, the present study was carried out with an aim to evaluate the role of AgNOR pleomorphism, morphometric studies and Ki-67 index as prognostic markers in gall bladder cancer.

Material and Methods:-

The study population comprised patients undergoing surgical procedure for gall bladder diseases either through open or laparoscopic procedures. This Prospective study was carried out at Department of Pathology in collaboration with Department of Surgery, Era's Lucknow Medical College and Hospital, Lucknow during year 2013 to 2015. Twenty five cases diagnosed and histologically confirmed of GB cancer was included in the study. Twenty five specimens from patients of non-neoplastic GB lesions were taken as controls. Patients with double malignancy, immunodeficiency diseases, or any other associated chronic debilitating disorder which is likely to interfere with detection of marker were excluded from the study.

Methodology:-

Surgically resected gallbladder specimen were grossed and processed including dehydration in increasing gradients of ethyl alcohol followed by cleaning in xylene and embedding in paraffin wax. Sections were cut and dewaxed and stained by H&E for routine histological diagnosis. Histopathological lesions were classified into neoplastic and non-neoplastic lesions. Histological grading was done in malignant cases into well, moderate, poorly differentiated, and other variants of carcinoma gall bladder. Cases were subjected for immunohistochemistry for Ki-67, AgNOR & morphometric analysis.

Immunohistochemical evaluation of ki67:-

Immunohistochemistry was performed on formalin fixed paraffin embedded tissue blocks with adequate tumor. IHC was done by streptavidin biotin method as per protocol standardized in our laboratory. Primary antibody and secondary antibody used for Ki67 was antibody (EPR 3611) from BIOGENEX, Fremont CA, USA & secondary antibody was Dako REAL EnVision, HRP RABBIT / MOUSE (ENV) from DAKO North America, Inc. Carpinteria CA USA respectively.

Sections of 3um were cut and taken on poly L – Lysine coated slides. Slides were dried for 16 hours at 37^{o}C followed by 1 hour at 60^{o}C. Sections were deparaffinised by heating on the slide warming table at 60^{o}c for 15-20 minutes and then passed through two changes of xylene for 5 minutes each. Sections were rehydrated by taking them through 3 changes of 99% alcohol for 5 minutes each, followed by 95% and 70% alcohol for 5 minutes each. Sections were then brought down to water for 10 minutes. Antigen retrieval was done by placing the slides in a coplin jar containing citrate buffer and processed in a microwave. Lid were placed on the coplin jars and heated to the maximum effect i.e. 800W till the time the fluid boils and then the microwave oven was set at mid effect i.e. 400W for 15 minutes. Containers were placed in a gentle water rinse for 5 minutes. Washed in Tris Buffer Saline (3 changes). Sections were treated with 3% H_2O_2 solution for 10 minutes to quench endogenous peroxidase activity. Washed in Tris Buffer Saline (3 changes) and incubated with primary antibody for 1 hour. Again washed with Tris Buffer Saline for (3 changes) and incubated with secondary antibody for 30 minutes. Sections were washed in 3 changes of Tris Buffer Saline (TBS) for 5 minutes each and then covered with freshly prepared DAB chromogen solution for 1 minute. The slides were then washed with water and counterstained with Harris hematoxylin for 1 minute, washed gently under running water. The sections were dehydrated, dipped in xylene bath and later were mounted using DPX, a non aqueous mounting medium. A positive and negative control was run with each batch of IHC staining.

Interpretation of IHC staining:-

The Ki67 staining reaction was considered positive only in the presence of immunostained nuclei in brown shades. A total of 25 microscopic fields were examined for Ki67 scoring. For each microscopic field, scoring was done using the following criteria (Giurgea et al., 2012) Quantitative assessment was done according to the number of stained cells:

- 0: score 0;
- 1-10%: score 1;
- 10-50%: score 2;
- 50-100%: score 3

For final score, the sum of 25 microscopic fields was taken in order to express the final score out of 100. The obtained score was expressed as % expression.

AgNOR staining & interpretation:-

AgNOR staining was performed on 3-4 mm thick sections from the paraffin block, placed on serum coated slides, and fixed at 37^{0}C overnight. Sections were then dewaxed in several baths of xylene and hydrate through graded alcohols 100% & 70% for 2-5 minute each to ultrapure water.

AgNORs staining reagent was prepared in the dark. Two solutions were needed: The first one (solution A) is a 2% gelatin solution dissolved in ultrapure water, to which formic acid is then added to make a final 1% solutions; the second one (solution B) was a 50% silver nitrate solution in ultrapure water. The staining solution was obtained instantly by rapidly mixing one part of solution A with two parts of solution B into a glass cylinder.

The staining solution (< 0.3 ml) was immediately poured on each slide. Staining takes 14-20 min at room temperature, depending on the desired intensity of the reaction. Direct sunlight was avoided during silver-staining. After staining, the solution was poured off and the slides were washed in several baths of ultrapure water, placed for 10 min in a 5% thiosulfate solution, washed again in several baths of ultrapure water, dehydrated, cleared with xylene, and mounted in DPX.

Quantitative analysis of AgNOR proteins:-
AgNORs appear as brown or black dots of various sizes within a yellowish background of nucleus. **AgNOR** counting included counting of each silver stained black color dots in the nucleus and nucleolus by light microscope under oil immersion lens at 100X magnification. In every section, 100 nuclei were examined in center of 10 fields. All the separate silver stained structures which could be clearly resolved were counted, whereas small dots overlapping each other were counted as one. The mean of the 100 nuclei were taken as the AgNOR index.

Morphometry:-

The quantitative morphometric studies were done by image analysis. Each 3-4 mm thick sections are cut from the paraffin block of the cases and controls were subjected to morphometric analysis using automated image analysis method on LEICA FLUORESCENT AND PHASE CONTRAST MICROSCOPE (GmBH) by a Leica Q Win is Lieca's Windows based image analysis toolkit running under the industry standard Microsoft windows environment. Area on slide to be imaged was visually selected with best morphological clarity & no overlapping. Cell diameter and nuclear diameter & N: C ratio was calculated. 100 cells were analysed in each target area of the microscopic field. The average of these was then taken.

The morphometric values of the above variables were compared between neoplastic and non-neoplastic gall bladder lesions and among different grades of malignant cases using t- test. A receiver operator curve analysis was performed to find out a suitable cut-off value of different morphometric parameters for differentiation between neoplastic and non-neoplastic gall bladder lesions.

Statistical analysis:-

Data so collected was subjected to Statistical Analysis using Statistical Package for Social Sciences, version 15.0. Chi-square test was used for proportions. Independent samples‘t’ test and ANOVA was used for comparison of mean values. Receiver-Operator Curve Analysis was performed to deduce a discriminant cut-off value of test variables. The confidence level of the study was kept at 95%, hence a ‘p’ value less than 0.05 indicated a statistically significant association.

Results:-

In this study of 50 cases, 25 were neoplastic and whereas remaining 25 were non-neoplastic cases (chronic cholecystitis). Among neoplastic cases, 15 were adenocarcinoma, 3 mucinous, 6 papillary and 1 signet ring carcinoma. Age of patients ranged from 30 to 70 years. Overall mean age of patients was 48.42±12.26 years. Majority of patients were aged below 40 years of age (n=27; 54%). Age of patients in neoplastic group ranged from 30 to 70 years with a mean age of 55.88±8.93 years. The proportion of patients aged above 50 years was 72%. In Non-neoplastic group, age of patients ranged from 31 to 63 years with a mean age of 40.96±10.55 years. The proportion of patients aged above 50 years was 20%. The difference between two groups was found to be statistically significant ($p<0.001$). (Table I)

Histopathological lesions were classified into neoplastic and non-neoplastic lesions. Histological diagnosis, grading in malignant cases into well, moderate, poorly differentiated, and other variants of carcinoma gall bladder was done with interpretation of immunohistochemistry for Ki-67, AgNOR & morphometric analysis. (Figure1, 2, 3)

On **morphometry**, all the three morphometric parameters, viz. cytoplasmic area, nuclear area and N/C ratio were higher in Neoplastic group as compared to Non-neoplastic group yet the difference between two groups was significant statistically only for nuclear area and N/C ratio ($p<0.05$) (Table II). A receiver operator curve analysis was performed to find out a suitable cut-off value of different morphometric parameters for differentiation between neoplastic and non-neoplastic gall bladder lesions (Graph I). The area under curve values for cytoplasmic area, nuclear area and N/C ratio were 0.542, 0.960 and 0.907 respectively, of which only nuclear area and N/C ratio values fulfilled the conditions of discriminant assessment (AUC>0.7). On exploring the data further, a cut-off value

of Nuclear area ≥86 was observed to be 96% sensitive and 84% specific. Similarly, a cut-off value ≥0.433 for N/C ratio was observed to be 100% sensitive and 84% specific. Mean N/C ratio of patients with well differentiated grade was 0.54±0.07 which was lower as compared to that of patients in moderate (0.61±0.12) and poorly differentiated (0.73±0.15) groups and the difference among different histological grades was significant (p=0.005). Among different types of tumors – mean value was minimum for Signet ring type and maximum for mucinous type. However, difference among different types was not significant statistically (p=0.267) (Table IV**).**

AgNOR count ranged from 4 to 14% in neoplastic group and 1 to 4% in Non-neoplastic group. Mean AgNOR count in neoplastic group was 7.88±2.42 as compared to 2.32±1.07 in Non-neoplastic group. On comparing the data statistically, the difference between two groups was found to be significant (p<0.001) (Table V). On carrying out a receiver-operator curve analysis for differentiation between non-neoplastic and neoplastic lesions based on AgNOR count, the area under curve value was 1.000, thus indicating an absolute discriminant value of the parameter. On exploring further, the best cut-off value obtained was ≥5.0% which was 100% sensitive and 100% specific in discriminating between neoplastic and non-neoplastic lesions. (Graph II, Table VI). Mean AgNOR count was higher in poorly differentiated grade (12.50±1.29) as compared to moderately differentiated (7.80±0.84) and well differentiated (6.75±1.34) grades. On evaluating the data statistically this difference was significant statistically (p<0.001). With respect to histopathological type, no significant difference could be seen among different types, though mean values in Adenocarcinoma group were higher (8.73±2.60) as compared to other types where mean value ranged from 6.00±1.00 to 7.00 (p=0.177). (Table VII)

Ki-67 immunostaining was carried out in neoplastic group only**.** Ki-67 expression ranged from 15 to 40% (Score 2) in neoplastic group including well differentiated, moderately differentiated and poorly differentiated adenocarcinoma. Mean expression was 28.20±7.83%. Among different histological grades, mean Ki-67 expression ranged from 30.50±8.54% (poorly differentiated) to 32.50±13.95% (well differentiated), however, this difference was not significant statistically (p=0.956). Mean Ki-67 expression for different types of neoplastic tumors ranged from 18.00 to 32.83±14.74%, however, this difference was not significant statistically (p=0.745). (Table VIII)

Discussion:-

The incidence of gall bladder diseases is increasing. Diseases of the gallbladder commonly manifest as gallstones and gallbladder cancer (Stanton et al., 2012). Although, gallbladder cancer is a rare yet it is associated with lethal malignancy with marked ethnic and geographical variations. Since the first description of the gallbladder carcinoma by Macmillan de Stool in 1777, studies have established a characteristic pattern of late diagnosis and ineffective treatment of this disease (Abi-Rached et al., 1995). The presenting symptoms are typically vague. Gallbladder cancer (GBC) can be clinically obvious, an unexpected finding at laparotomy, detected incidentally on histologic examination or may be missed only to present with recurrence during follow-up. GBC is characterized by local invasion, extensive regional lymph node metastasis, vascular encasement, and distant metastases. In general, GBC is the most aggressive of the biliary cancers with the shortest median survival duration (Kapoor, 2006). Owing to vague presentation their diagnosis commonly occurs at an advanced stage. This late diagnosis plus the anatomic feature that the gallbladder lacks a submucosa culminates in a rather dismal prognosis(Henson et al.,1992, Wistuba et al., 2004, Lai et al., 2008)

Considering these diagnostic difficulties and difficulty in staging of gall bladdercancer, it is essential that diagnostic techniques with adequate accuracy in terms of evaluation of malignancy status, staging and prognosis should be explored and developed . With this background the present study was carried out to correlate and evaluate the prognostic significance of the expression of AgNOR parameters, morphometry and Ki-67 index in neoplastic and non-neoplastic gall bladder lesions.

AgNORs have also been considered as an important prognostic factor in various malignant tumors (Gupta et al.,2005, Suzuki T et al., 1993;Misra V et al., 1995), similarly morphometric changes, specially N/C ratio and nuclear area have shown to be associated with proliferative activit. Ki-67 labeling index has been shown a good correlation with histological differentiation (Nakajo et al., 1989). In present study, a total of 50 cases with suspicious gall bladder pathology were enrolled – of these 25 (50%) had neoplastic lesions while remaining 25 (50%) were cases of chronic cholecystitis. As neoplastic lesions of gall bladder often have non-specific clinical features are mimic the clinical picture and presentation similar to that of patients with cholelithiasis. Although among gall bladder diseases, neoplastic or malignant lesions are rare however, the sample distribution of two groups was kept similar in order to maintain adequacy of samples in both groups.

With respect to different types of neoplastic lesions enrolled in the study, 15 (60%) were adenocarcinoma, 3 (12%) mucinous, 6 (24%) papillary and 1 (4%) signet ring carcinoma respectively. These findings are in agreement with the profile of gall bladder carcinoma described in literature. According to Shaffer (2008) most (>80%) gallbladder cancers are adenocarcinomas. Li et al. (2011) were of the view that more than 85% of gall bladder carcinomas belong to adenocarcinomas. In another study Mishra et al. (1995) found 40 of their 49 neoplastic gall bladder lesions (81.6%) to be adenocarcinoma. Yadav et al. (2013) in their study found the proportion of adenocarcinoma to be 86.7%. Thus, all these studies show adenocarcinoma to be the most frequent type of neoplastic lesions of gall bladder. In present study, papillary adenocarcinoma was found to be 24% which is relatively higher than the reported prevalence in different studies. Henson et al. (1992) reported their prevalence to be 5.8%. Yadav et al. (2013) reported the prevalence of papillary adenocarcinoma in their case series to be 9.4%. The relatively higher prevalence of papillary type in present study could be incidental and cannot be conclusive in a series of 25 cases only.

In present study, age of patients ranged from 30 to 70 years. However, majority of neoplastic cases were above 50 years of age (72%) whereas majority of non-neoplastic cases were $\leq$50 years of age (80%). This is in agreement with the observations of previous observation that risk of gall bladder cancer increases with advancing age. In present study, it was 55.88 years as compared to a median age of 67 years as reported by Duffy et al. (2008). Thus mean age of patients with neoplastic lesions was relatively lower in present study. In another study, Wang et al. (2015) reported the average age of their patients with gall bladder carcinoma to be 66.0 years. Henson et al. (1992) also reported the average age of their patients to be 72.2 years. One of the reasons for this could be a high number of cases having gall bladder carcinoma along with gall stone disease. This is in agreement with the observation of Dutta et al. (2005) who found that risk of gall bladder cancer is increased in younger patients with gall stone disease.

For both neoplastic as well as non-neoplastic lesion types, females predominated over males. In present study, male to female ratio of gall bladder cancer cases was 0.04:1. Although most of the studies have reported a high prevalence of females over males, however, the male to female ratio was variable in different studies. Henson et al. (1992) in their study found this ratio to be 0.36:1, Yadav et al. (2013) and Wang et al. (2015) reported this ratio to be 1:2. In a study by Hariharan et al. (2008) covering the data from 50 countries, gall bladder cancer was found to be affecting more women than men. In another population-based study in the rural Gangetic basin of north India, Unisa et al. (2011) reported prevalence of gall stone disease in higher number of females as compared to males with male to female ratio ranging from 0.83:1 among symptomatic to 0.31:1 among asymptomatic patients.

In present study, for the morphometric analysis was done by calculating cytoplasmic area, nuclear area and N/C ratio respectively. On comparing the cytoplasmic area between neoplastic and non-neoplastic groups, no significant difference was observed between two groups. However, both nuclear area as well as N/C ratio was found to be significantly higher in neoplastic group as compared to non-neoplastic group. The malignant cell is characterized by: acceleration of the cell cycle; genomic alterations; invasive growth; increased cell mobility; chemo taxis; changes in the cellular surface; secretion of lyric factors, etc. Morphologically, the cancerous cell is characterized by a large nucleus, having an irregular size and shape, the nucleoli are prominent, and the cytoplasm is scarce and intensely colored or, on the contrary, is pale. The nucleus of neoplastic cells plays through its changes a main role in the assessment of tumor malignancy. Changes concern its surface, volume, the nucleus/cytoplasm ratio, shape and density, as well as structure and homogeneity (Baba et al., 2007).

The findings in present study are similar to those obtained by Sen. et al. (2015) who also showed that except for cytoplasmic area for the entire parameters malignant group had significantly higher mean value as compared to those in benign group. Similar to results of present study, Nakajo et al. (1989) found that N/C ratio and nuclear area were significantly higher in gall bladder carcinoma cases as compared to controls. In another study (Nakajo et al 1990), they showed that N/C ratio in the met plastic type of both adenoma and adenocarcinoma was significantly larger than that in the non-met plastic type. In case series including lesion types other than gall bladder too, both N/C ratio and nuclear area were seen to be significantly higher in malignant type as compared to benign type (Rashid et al., 2009; Malhotra et al., 2013; Mihalache et al., 2014). Thus findings in present study support the view point that malignant cells are characterized by larger nuclear sizes and nuclear/cytoplasmic ratios as also supported in previous studies (Rashid et al.,2009; Malhotra et al.,2013; Mihalache et al., 2014) .

In neoplastic group, Ki-67 immunostaining was performed which showed an expression to the range of 15 to 40% with a mean expression of 28.20±7.83%. Ki-67 is considered to be a proliferative marker. Ki67 is a frequently examined proliferation marker with prognostic value in several carcinomas (Xuan et al., 2005). Ki67 has been reported as a prognostic parameter in squamous cell carcinomas of other origins such as laryngeal carcinoma, cervix carcinoma and oral carcinomas (Matsumoto et al., 1999; Valente et al., 1999; Padovan et al., 2000; Lazaris et al., 2002).It is seen to be positive in almost all malignant cases whereas in benign or normal cases it is either negative or mildly expressed (Xuan et al., 2005). Its positivity in malignant cases is reported to vary across in different case series. In present study, mean Ki-67 expression in neoplastic group was 28.20±7.83%, Takei et al. (1997) reported its positivity to be 36.6±5.6% in pure carcinomas of gall bladder. They also found its expression to be 12.5±1.9% in the carcinoma area of 16 tumors with carcinoma-in-adenoma, 7.9±1.7% and in the adenoma areas of 16 tumors with carcinoma-in-adenoma and 4.9±0.5% in the 32 pure pyloric gland adenomas. Hui et al. (2002) also reported expression of Ki-67 in 37 gallbladder carcinoma cases. Although some researchers like Sai et al. (2005) have evaluated the role of Ki-67 as a discriminant between benign and malignant cases of gall bladder lesions, however, in present study no such discriminant role was evaluated and its expression was limited in neoplastic cases only considering it to be a proliferative marker only.

With respect to AgNOR expression, the present study showed mean AgNOR levels to be in neoplastic as compared to non-neoplastic group (7.88±2.42 vs 2.32±1.07; $p<0.001$). It is another useful tumor proliferation marker (Janmohamed et al., 1990; Sakr et al., 1993; Itoi T et al., 2003; Garg et al.,2013). and has also proven to be a useful tool in staging of disease and in distinction between benign and malignant lesions. They also have a prognostic role. In a study by Suzuki et al. (1993) mean AgNOR count was found to be higher in gall bladder carcinoma cases as compared to normal gall bladder cases. In present study, although the comparison was made between neoplastic and non-neoplastic groups, a similar trend as observed by Suzuki et al. (1993) was obtained. In another study, Mishra et al. (1995) showed an incremental trend for mean AgNOR count starting from 1.97±0.28 (Chronic cholecystitis) to 5.6±0.88 (dysplasia) and finally reached at 8.5±0.78 (poorly differentiated adenoacarcinoma), thus showing a differential role of AgNOR between different types of gall bladder lesions. Similar to our study, Nanashima et al. (2002) also showed mean AgNOR count in malignant cases to be significantly higher (9.2±3.5) as compared to those with benign biliary disease (4.1±1.0 and 2.9±0.8). The findings in present study were also supported by the observations made by Gupta et al. (2013).

In present study, receiver operator curve analysis was performed to evaluate the diagnostic efficacy and discriminant role of cytoplasmic area, nuclear area and N/C ratio for differentiating between neoplastic and non-neoplastic lesions. The area under curve value above 0.7 was obtained only for nuclear area and N/C ratio. On exploring the data further, a cut-off value of Nuclear area $\geq$86 was observed to be 96% sensitive and 84% specific. Similarly, a cut-off value $\geq$0.433 for N/C ratio was observed to be 100% sensitive and 84% specific. Similar to our study Sen et al. (2015) also showed a high diagnostic efficacy (sensitivity 98.6% and specificity 94%) for nuclear area at a cut-off 88.30 and N:C ratio $\geq$0.345 (sensitivity 93% and specificity 94%), a finding similar to our study. In their study, cytoplasmic area was also found to have a good discriminant ability (sensitivity 100%, specificity 90%), however, in present study cytoplasmic area did not show any discriminant role. The reason for this could be the selection of different types of specimen for assessment. In present study, the assessments were performed on tissue sections whereas in the study of Sen et al. (2015) assessments were made in effusion specimen.

A similar exercise was also performed to deduce the discriminant efficiency of AgNOR. In present study, AgNOR was found to have a highly effective and efficient discriminant role with 100% sensitivity and 100% specificity at a cut-off $\geq$5%. AgNOR count has a high discriminant efficacy for differentiation between benign and malignant cases. In present study, it was 100% accurate at a cut-off $\geq$5% whereas Nanashima et al. (2002) showed the 80% sensitivity and 100% specificity at a cut-off value of 7, i.e. a result similar to that obtained in present study.

In present study, N/C ratio showed a significant increment from well differentiated (0.54±0.07) to moderately differentiated (0.61±0.12) and poorly differentiated (0.73±0.15) histological grades. However, no significant association between N/C ratio and different histopathological types of gall bladder carcinoma was observed. None of the studies on gall bladder lesions have studied this relationship, however, keeping in view the suggested role of skewed N/C ratio to be an indicator of a proliferative activity this association could be perceived to hold good. With respect to absence of a significant association between N/C ratio and histopathological type, it could be attributed to be less number of cases with different histopathological types.

In present study Ki-67 labeling index was also seen to be increasing with from well (25.50±6.73%) to moderately (29.40±8.36%) and poorly (37.50±3.77%) differentiated histopathological types. This finding is in agreement with the findings in literature that also found a similar association and once again highlighted the role of Ki-67 as a proliferation marker.

For AgNOR count too, a significant increment was observed from well differentiated (6.74±1.34) to moderately differentiated (7.80±0.84) and poorly differentiated (12.50±1.29) histological grades. However, no significant association between AgNOR count and different histopathological types of gall bladder carcinoma was observed. The findings indicated that AgNOR is a proliferating marker which helps to differentiate among different grades. A similar incremental pattern with decreasing grade of differentiation of adenocarcinoma was observed by Gupta et al. (2003). In another study, Gupta et al. (2013) showed a similar discriminate role of AgNOR count.

In present study, no significant difference in mean AgNOR count for different histopathological types. None of the studies reviewed by us showed a significant role of AgNOR in differentiating different histopathological types. One of the reasons for this could be a high prevalence of adenocarcinoma and relatively less representation of other types in almost all the studies.

The findings of present study thus showed that AgNOR count, cytomorphometric and Ki-67 index have a significant role in differentiating non-neoplastic from neoplastic types of lesions. Moreover, these features also helped to differentiated amongst different histopathological grades. One of the limitations of the study was small sample size and very little number of cases with different histopathological types. Unfortunately, owing to rarity of gall bladder carcinoma, there are limited studies evaluating the role of these parameters despite their showing a promising role in differentiating neoplastic from non-neoplastic lesions and differentiation of different histopathological grades of gall bladder carcinoma, owing to this limitation, the evidence related with these associations needs an empirical validation for which further studies are recommended.

Figure 1: Well differentiated adenocarcinoma Gallbladder; 1(a)H&E, 40X; 1(b) Morphometry (nuclear & cytoplasmic diameter), H&E,400X; 1(c)IHC for Ki67, 100X; 1(d) Nuclear AgNOR dots ,AgNOR stain, 630X)

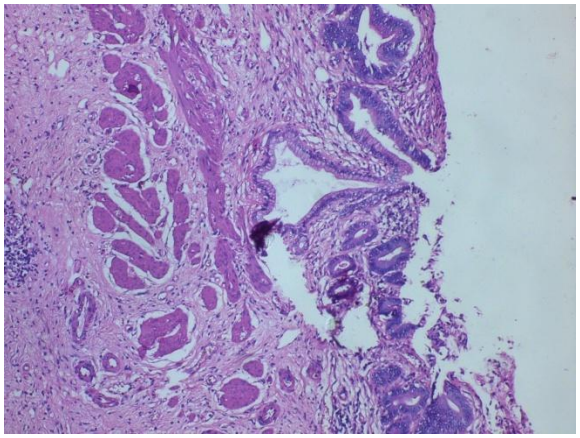


Figure 1a

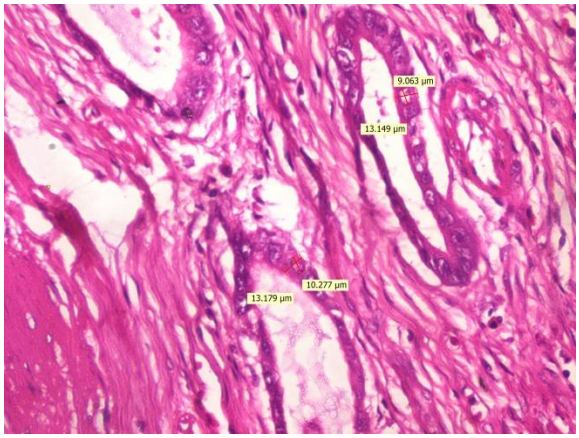


Figure 1b

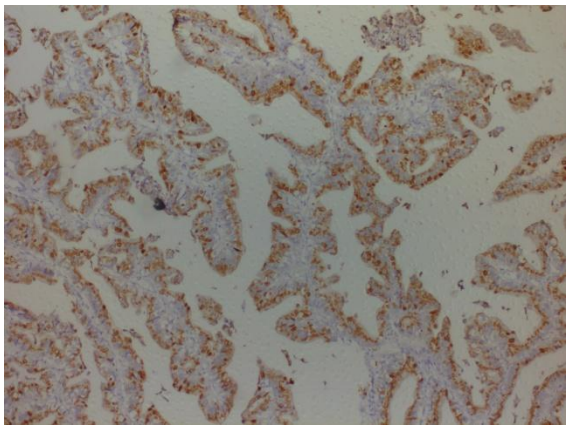


Figure 1c

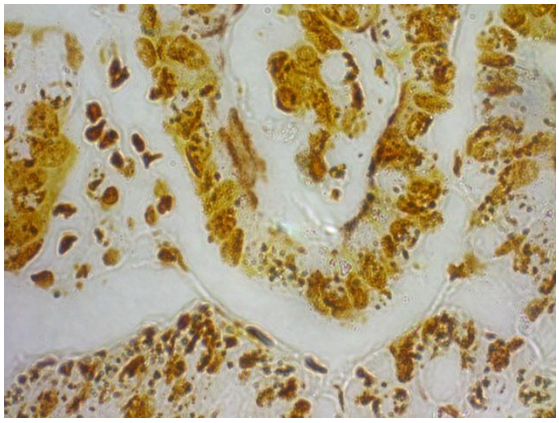


Figure 1d

Figure 2: Moderately differentiated adenocarcinoma Gallbladder; 2(a) Morphometry (nuclear & cytoplasmic diameter), H&E, 400X; 2(b) Nuclear AgNOR dots, AgNOR stain, 630X)

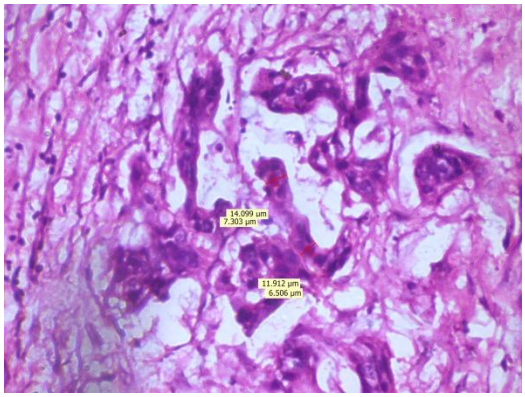


Figure 2a

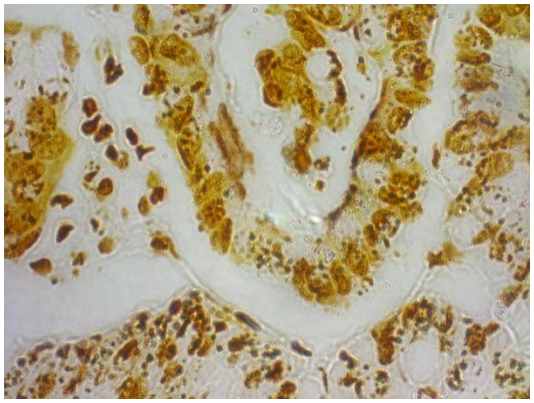


Figure 2b

Figure 3: Poorly differentiated adenocarcinoma Gallbladder; 3(a) H&E, 400X; 3(b) IHC for Ki67, 400X.

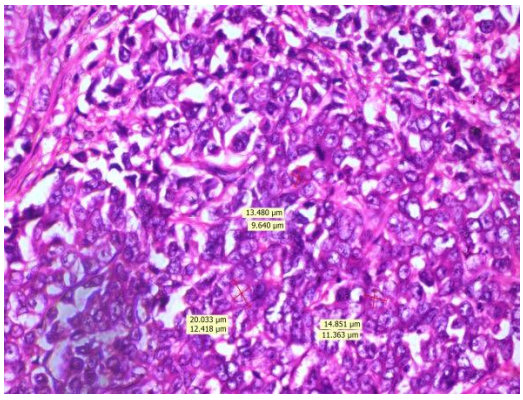


Figure 3a

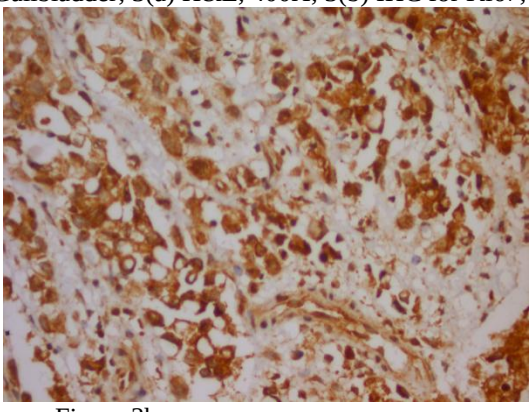


Figure 3b

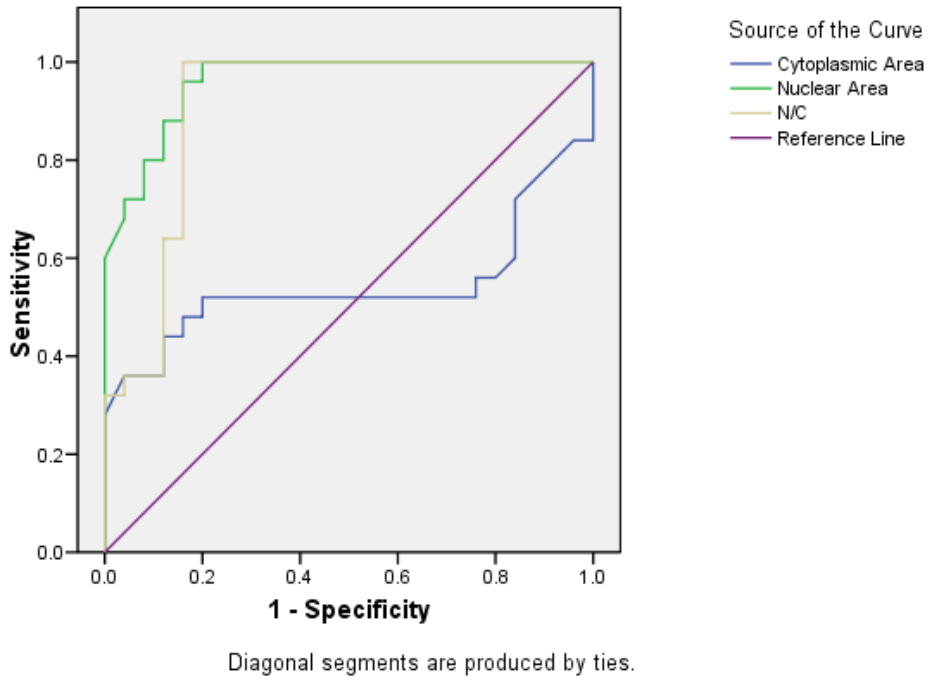
Table I: Distribution of Gall bladder lesions according to Age & Sex

Age wise distribution	Neoplastic (n=25)		Non-neoplastic (n=25)	
	No.	%	No.	%
30-40 Yrs	1	4	13	52
41-50 Yrs	6	24	7	28
51-60 Yrs	12	48	2	8
61-70 Yrs	6	24	3	12
Mean Age±SD (Range)	55.88±8.93 (30-70)		40.96±10.55 (31-63)	
χ^2=18.505 (df=3); p<0.001 (S)				
Sex wise distribution Total n = 50				
Male N=4	1	4	3	12
Female N = 46	24	96	22	88
χ^2=1.087 (df=1); p=0.297 (NS)				

Table II: Comparison of Morphometric Characteristics between neoplastic and non-neoplastic lesions

S. No.	Morphometry	Neoplastic group (n=25)		Non-neoplastic group (n=25)		Significance	
		Mean	SD	Mean	SD	't'	'p'
1.	Cytoplasmic area (µm²)	205.4	30.8	199.7	11.2	0.86	0.394
2.	Nuclear area (µm²)	117.4	17.3	70.1	18.2	9.41	<0.001
3.	N/C	0.58	0.12	0.39	0.10	6.15	<0.001

Derivation of cut-off value of morphometric parameters for differentiation between neoplastic and non-neoplastic lesions.



Graph I : Receiver operator curve analysis in gall bladder lesion for morphometry.

Table III: Area under the Curve

Test Result Variable(s)	Area	Std. Error[(a)]	Asymptotic Sig.[(b)]	Asymptotic 95% Confidence Interval	
				Lower	Upper
Cytoplasmic Area	0.542	0.090	0.607	0.365	0.720
Nuclear Area	0.960	0.024	<0.001	0.914	1.006
N/C	0.907	0.046	<0.001	0.816	0.998

The test result variable(s): Cytoplasmic Area, Nuclear Area has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
a Under the nonparametric assumption
b Null hypothesis: true area = 0.5

Table IV: Association of morphometric variable N/C ratio with grade and histopathological type of gall bladder cancer.

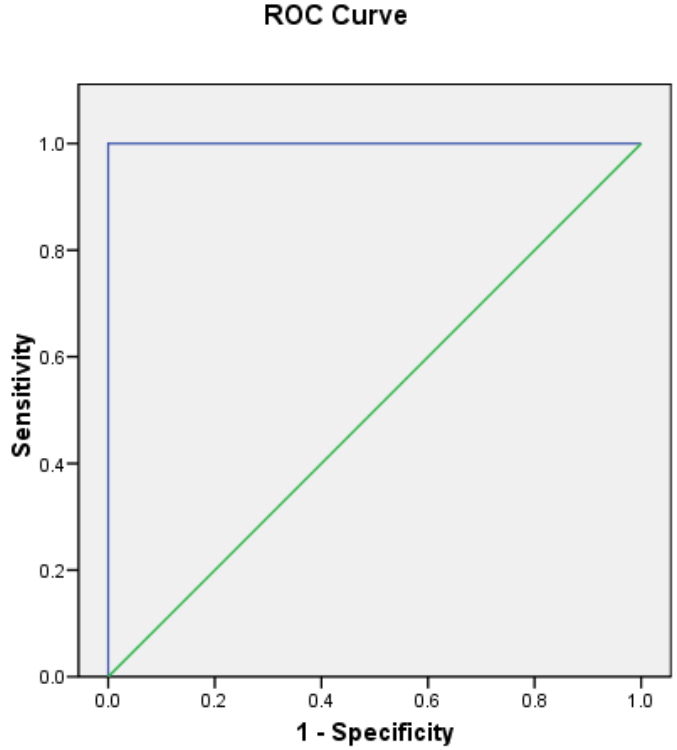
Variable	N	Mean N/C ratio	Std. Deviation	Significance
Grade (N=25)				
Well diff.	16	0.54	0.07	F=6.756; p=0.005
Mod. diff.	5	0.61	0.12	
Poorly diff.	4	0.73	0.15	
Type (N=25)				
Adenocarcinoma	15	0.60	0.13	F=1.170; p=0.345
Mucinous	3	0.64	0.01	
Papillary	6	0.52	0.06	
Signet	1	0.49		

Table V: Comparison of AgNOR count between neoplastic and Non-neoplastic groups

Group	N=50	Mean AgNOR count	Std. Deviation	Minimum	Maximum
Neoplastic	25	7.88	2.42	4	14
Non-neoplastic	25	2.32	1.07	1	4
Total	50	5.26	3.16	1	11

t=10.505; p<0.001

Derivation of cut-off value of AgNOR count for differentiation between neoplastic and non-neoplastic lesions.



Graph II: Receiver operator curve analysis in gall bladder lesion for AgNOR count.

Table VI: Area under the Curve

	Std. Error(a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
1.000	.000	<0.001	1.000	1.000

a Under the nonparametric assumption
b Null hypothesis: true area = 0.5

Table VII: Association of AgNOR with grade and histopathological types of gall bladder cancer

Variable	N	Mean	Std. Deviation	Significance
Grade				
Well diff.	16	6.75	1.34	F=33.45; p<0.001
Mod. diff.	5	7.80	0.84	
Poorly diff.	4	12.50	1.29	
Type				
Adenocarcinoma	15	8.73	2.60	F=1.808; p=0.177
Mucinous	3	6.00	1.00	
Papillary	6	6.83	1.72	
Signet	1	7.00		

Table VIII: Association of Ki-67 expression with grade and histopathological types of gall bladder cancer

Variable	N	Mean	Std. Deviation	Significance
Grade				
Well diff.	16	25.50	6.73	F=5.156; p=0.015
Mod. diff.	5	29.40	8.36	
Poorly diff.	4	37.50	3.77	
Type				
Adenocarcinoma	15	29.80	7.93	F=0.878; p=0.468
Mucinous	3	26.33	5.69	
Papillary	6	26.83	8.52	
Signet	1	18.00		

Conclusion:-

On the basis of above evaluation, it can be concluded that AgNOR count, Ki-67 and cytomorphometric parameters i.e. N/C ratio show a high discriminant value in discrimination between neoplastic and non-neoplastic lesions. These markers also showed a significant association with the histopathological grade, however, owing to less number of cases, the association between histopathological type and these markers remains unsubstantiated. Further studies on larger sample size are recommended.

References:-

1. Abi-Rached B, Neugut AI. Diagnostic and management issues in gallbladder carcinoma. Oncology. 1995; 9:19–30.
2. Ahsan S., Tayyab M., Chaudhry N.A., Khan S.A; Silver staining nucleolar organizer regions (AgNOR) typing in nodular hyperplasia and carcinoma of the prostate PPM J 1991-92; 2-3(1-4, 1-2): 67-72.
3. Anastassova-Kristeva M; The nucleolar cycle in man. J Cell Sci 1977;25:103-10
4. Anselmi L, Sementa AR, Borgiani L, Banderali A; Rovida S. Nucleolar organiser regions (NOR) in normal and pathological liver; a quantitative analysis. Pathol 1990; 82(1082): 653-54.
5. Baba AI, Câtoi C. Comparative Oncology. Bucharest: The Publishing House of the Romanian Academy; 2007. Chapter 3, TUMOR CELL MORPHOLOGY.
6. Bernardi FDC, Capelozzi VL, Takagaki TY, Younes RN, Saldiva PHN;Usefulness of morphometric evaluation of histopathologic slides in predicting long-term outcome of patients with squamous cell carcinoma of the lung; A preliminary report. Chest 1995; 107:614-20.
7. Chen A, Huminer D.: The role of estrogen receptors in the development of gallstones and gallbladder cancer. Med Hypotheses 36:259-260, 1991.
8. Dhir V, Mohandas KM.: Epidemiology of digestive tract cancers in India IV, gallbladder and pancreas. Indian J Gastroenterol 18:24-28, 1999.
9. Doval DC, Azam S, Sinha R, Batra U, Mehta A. Expression of epidermal growth factor receptor, p53, Bcl2, vascular endothelial growth factor, cyclooxygenase-2, cyclin D1, human epidermal receptor-2 and Ki-67: Association with clinicopathological profiles and outcomes in gallbladder carcinoma. J Carcinog 2014;13:10.
10. Duffy A, Capanu M, Abou-Alfa GK, et al. Gallbladder cancer (GBC): 10-year experience at Memorial Sloan-Kettering Cancer Centre (MSKCC). J Surg Oncol. 2008; 98(7):485–489.
11. Dutta U, Nagi B, Garg PK, Sinha SK, Singh K, Tandon RK. Patients with gallstones develop gallbladder cancer at an earlier age. Eur J Cancer Prev. 2005 Aug;14(4):381-5.
12. Field DH, Fitzgerald PH, Sin FYT. Nucleolar silver-staining patterns related to cell cycle phase and cell generation of PHA-stimulated human lymphocytes. Cytobios; 1984; 41:23-33.
13. Garg KN, Raj V, Chandra S. Evaluation of the efficacy of AgNOR as a proliferative marker in oral leukoplakia: A morphometric analysis. Natl J Maxillofac Surg. 2013 Jan-Jun; 4(1): 40–45.
14. GIURGEA LN, UNGUREANU C, MIHAILOVICI MC. The immunohistochemical expression of p53 and Ki67 in ovarian epithelial borderline tumors. Correlation with clinicopathological factors. Rom J Morphol Embryol. 2012; 53(4):967–973

15. Goodpasture C, Bloom SE. Visualisation of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma 1975; 53:37-50.
16. Gupta N, Kaur A, Pal S, Kahlon SK. Study of nucleolar organizer regions (NORs) in lesions of gallbladder. Indian J Pathol Microbiol. 2003 Jul; 46(3):371-4.
17. Gupta S, Misra S, Goyal MM, Kumar V, Chaturvedi A, Arya NK, Jain J, Akhtar N. Prognostic significance of AgNOR Proliferative Index in gallbladder carcinoma - A potential alternative to frozen section analysis. J Liver 2014; 3: 143. doi:10.4172/2167-0889.1000143.
18. Gupta SK, Shukla VK. Gall bladder cancer. Etiopathology and Treatment. Health Administrator 2005; 17I (1):145-142.
19. Henson DE, Abores-Saavendra J, Corle D. Carcinoma of the gallbladder. Histologic types, stage of disease, grade, and survival rates. Cancer. 1992 Sep 15; 70(6):1493-7.
20. Henson DE, Albores-Saavedra J, Corle D. Carcinoma of the gallbladder. Histologic types, stage of disease, grade, and survival rates. Cancer. 1992; 70: 1493–1497.
21. Hofgartner FJ, Krone W, Jain K. Correlated inhibition of ribosomal RNA synthesis and silver staining by actinomycin D. Hum Genet 1979;47:329-33.
22. Hui AM, Shi YZ, Li X, et al Proliferative marker Ki-67 in gallbladder carcinomas: high expression level predicts early recurrence after surgical resection. Cancer Lett 2002;176: 191-8.
23. Janmohamed RMI, Murray PG, Crocker J, Leyland MJ. Sequential demonstration of nucleolar organizer regions and Ki67 immunolabelling in non-Hodgkin's lymphomas. Clin Lab Haematol 1990; 12:395–399.
24. Kapoor VK. Gallbladder cancer: A global perspective. J Surg Oncol. 2006; 93:607–9.
25. Khanduri S et al., Morphometry in Pediatric Neoplasms; Department of pathology, C.S.M. University, Lucknow. August, 2003.
26. Lai CH, Lau WY. Gallbladder cancer: a comprehensive review. Surgeon 2008; 6: 101–110.
27. Lazaris AC, Rigopoulou A, Tseleni-Balafouta S, Kavantzas N, Thimara I, Zorzos HS, Eutychiadis CA, Petraki K, Kandiloros D, Davaris P. Immunodetection and clinico-pathological correlates of two tumor growth regulators in laryngeal carcinoma. Histol. Histopathol. 2002; 17: 131-138.
28. Li Q-L, Yang Z-L, Liu J-Q, Miao X-Y. Expression of CDX2 and Hepatocyte Antigen in Benign and Malignant Lesions of Gallbladder and Its Correlation with Histopathologic Type and Clinical Outcome. Pathology Oncology Research. 2011; 17(3):561-568.
29. Macartney JC, Smith PJ. Correlation between DNA flow cytometric and nucleolar organizer region data in non-Hodgkin lymphomas. J Pathol 1988; 154:151 -6.
30. Malhotra S, Kazlouskaya V, Andres C, Gui J, Elston D. Diagnostic cellular abnormalities in neoplastic and non-neoplastic lesions of the epidermis: a morphological and statistical study. Journal of Cutaneous Pathology. 2013; 40(4):10.1111/cup.12090. doi:10.1111/cup.12090.
31. Matsumoto M, Komiyama K, Okaue M, Shimoyama Y, Iwakami K, Namaki S, Tanaka H, Moro I, Sato H. Predicting tumor metastasis in patients with oral cancer by means of the proliferation marker Ki67. J. Oral. Sci. 1999; 41, 53-56.
32. Mihalache D, Giuşcă S, Balan R, Amălinei C, Grigoras A, Căruntu I-D. A morphometric approach in breast cytology – geometrical descriptors in the differentiation between benign and malignant lesions. Rom J Morphol Embryol 2014, 55(2):273–277.
33. Misra V, Misra SP, Dwivedi M, Gupta SC. Role of AgNORs in diagnosis of early malignant lesions of gall bladder. Indian J Pathol Microbiol. 1995 Oct; 38(4):383-8.
34. Morton CC, Brown J A, Holmes WM, Nance WE, Wolf B;Stain intensity of human nucleolus organizer region reflects incorporation of uridine into mature ribosomal;RNA. Exp Cell Res 1983; 145:405-13.
35. Mulazim Hussain Bukhari, Shahida Niazi, Ihsanullah Hashmi, Samina Naeem, Abdul Khalik Abro, Mohammad Tayyab, Naseer Ahmad Chaudhry; The importance of AgNOR staining in grading and differential diagnosis of Astrocytic lesions;2007April-june;23(2)
36. Nakajo S, Yamamoto M, Tahara E. Morphometrical analysis of gall-bladder adenoma and adenocarcinoma with reference to histogenesis and adenoma-carcinoma sequence. Virchows Archiv A 1990; 417 (1): 49-56.
37. Nakajo S, Yamamoto M, Tahara E. Morphometric analysis of gallbladder adenocarcinoma: discrimination between carcinoma and dysplasia. Virchows Arch A Pathol Anat Histopathol. 1989; 416(2):133-40.
38. Nanashima A, Yamaguchi H, Nishizawa-Takano JE, Hatano K, Shibasaki S, Sawai T, Yasutake T, Obatake M, Nakagoe T, Ayabe H. Application of argyrophilic nucleolar organizer region (AgNOR) staining for cytology of biliary tract carcinomas. J Hepatobiliary Pancreat Surg. 2002; 9(4):485-9.
39. National Cancer Registry Programme, Consolidated reports of the population based cancer registries 1990-96, Indian Council of Medical Research, New Delhi, India, August 2001.

40. Ploton D, Menager M, Adnet J-J. Simultaneous high resolution of Ag-NOR proteins and nucleoproteins in interphasic and mitotic nuclei. Histochem J 1984; 16: 897-906.
41. Rashid F, Haque AU. Nuclear Morphological and Morphometric Features of Reactive versus Neoplastic Gastric Mucosa. International Journal of Pathology; 2009; 7(2): 66-72.
42. Raymond WA, Leong AS. Nucleolar organizer regions relate to growth fractions in human breast carcinoma; Hum Pathol 1989; 20:741-6.
43. Roa E I, Elorza D X, Lantadilla H S, Ibacache S G, Aretxabala U Xd.[Immunohistochemical expression of Ki-67 as a marker of proliferation in gallbladder mucosa samples with or without cancer]. Rev Med Chil. 2009 Jul; 137(7):881-7. Epub 2009 Sep 24.
44. Sai JK, Suyama M, Nobukawa B, Kubokawa Y, Yokomizo K, Sato N. Precancerous mucosal changes in the gallbladder of patients with occult pancreatobiliary reflux. Gastrointestinal Endoscopy 2005; 61(2): 264-268.
45. Sakr WA, Sarkar FH, Sreepathi P, Drozdowicz S, Crissman JD. Measurement of cellular proliferation in human prostate by AgNOR, PCNA, and SPF. The Prostate 1993; 22(2): 147-154.
46. Scholzen T, Gerdes J (March 2000). "The Ki-67 protein: from the known and the unknown". J. Cell. Physiol. 182 (3):311-22.
47. Schottenfeld D and Fraumeni J. Cancer. Epidemiology and Prevention. 3rd. Oxford University Press; 2006:787-800.
48. Sen R, Hasija S, Kalra R, Garg S, Singh A, Megha. Morphometric Analysis and Immunocytochemical Staining on Cytospin Preparation in Effusion Cytology: A Study. Cytol Histol 6: 314. doi:10.4172/2157-7099.1000314.
49. Shaffer EA. Gallbladder Cancer: The Basics. Gastroenterol Hepatol (N Y). 2008 Oct; 4(10): 737–741.
50. Shahida Parveen, M. H. Bukhari, S. Akhtar Khan, I. A. Naveed, N. A. Chaudhry and M. Tahseen; AgNOR stain in normal, cirrhotic and carcinomatous liver; Biomedica Vol. 22 Jan. - Jun. 2006/Bio-l 7.
51. Shrestha ML, Miyake H, Kikutsuji T, Tashiro S. Prognostic significance of Ki-67 and p53 antigen expression in carcinomas of bile duct and gallbladder. J Med Invest. 1998 Aug; 45(1-4):95-102.
52. Siddiqui MS, Soomro IN, Kayani N, Muzzafar S, Hasan SH;Assessment of nucleolar organiser regions (NORs) in proliferative conditions. Pathol;Res Practl999; 194 (6): 421-426
53. Singletary BK, Van Thiel DH, Eagon PK.: Estrogen and progesterone receptors in human gallbladder. Hepatology 6:574-578, 1986.
54. Stinton LM, Shaffer EA. Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. Gut and Liver. 2012; 6(2):172-187.
55. Suzuki T, Takano Y, Kida Y, Okudaira M. Silver-binding argyrophilic nucleolar organizer regions (AgNOR) in gall-bladder cancer: Correlations with malignant grading and prognosis. Pathology International 1993; 43(1-2): 36-43.
56. Takei K, Watanabe H, Itoi T, Saito T. p53 and Ki-67 immunoreactivity and nuclear morphometry of 'carcinoma-in-adenoma' and adenoma of the gall-bladder. Pathol Int. 1996 Jun; 46(6):426-35.
57. Tsuchida A, Itoi T, Aoki T, Koyanagi Y. Carcinogenetic process in gallbladder mucosa with pancreaticobiliary maljunction (Review). Oncol Rep. 2003 Nov-Dec; 10(6):1693-9.
58. Unisa S, Jagannath P, Dhir V, Khandelwal C, Sarangi L, Roy TK. Population-based study to estimate prevalence and determine risk factors of gallbladder diseases in the rural Gangetic basin of North India. HPB : The Official Journal of the International Hepato Pancreato Biliary Association. 2011; 13(2):117-125.
59. Wang J-H, Liu B-J, Xu H-X, et al. Clinical, pathological and sonographic characteristics of unexpected gallbladder carcinoma. International Journal of Clinical and Experimental Medicine. 2015; 8(7):11109-11116.
60. Weibel E. R., Stereological Methods: Vol. 1; Practical Methods for Biological Morphometry, London, Academic Press, 1979.
61. Wistuba II, Gazdar AF. Gallbladder cancer: lessons from a rare tumor. Nat Rev Cancer. 2004; 4: 695–706.
62. Xuan YH, Choi YL, Shin YK, Kook MC, Chae SW, Park SM, Chae HB, Kim SH. An immunohistochemical study of the expression of cell-cycle-regulated proteins p53, cyclin D1, RB, p27, Ki67 and MSH2 in gallbladder carcinoma and its precursor lesions. Histol. Histopathol. 2005; 20: 59-66.
63. Yadav R, Jain D, Mathur SR, Sharma A, Iyer VK. Gallbladder carcinoma: An attempt of WHO histological classification on fine needle aspiration material.CytoJournal. 2013; 10:12.