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The Use of Flow Cytometric DNA Ploidy Analysis of Liver Biopsies in Liver Cirrhosis and Hepatocellular Carcinoma

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common human cancer world-wide but the third most common cause of mortality and evidence has been accumulating in various countries that HCC is increasing (Andrisani et al., 2011; Parkin et al., 2000; Perkin, 2004). Major etiologic agents in HCC pathogenesis are chronic infection with hepatitis B virus or hepatitis C virus (Bruix et al., 2004; Poynard et al., 2003). Advanced liver fibrosis and HCC have been indicated to develop in about 30% of patients with chronic hepatitis B or C. Other causal factors of lower incidence include alcohol abuse, metabolic disorders, and environmental agents, e.g., exposure to aflatoxin B1 (Llovet et al., 2003). HCC typically develops in the cirrhotic liver in about 80% of cases and develops only in the non-cirrhotic liver in less than 20% of cases (Alkofer, 2011). When diagnosed at early stage, HCC remains eligible for potential curative options such as surgical resection, orthotopic liver transplantation or percutaneous destructions. The absence of advanced fibrosis or cirrhosis makes resection feasible more often. However, most of HCCs have widespread dissemination within the liver at diagnosis (intermediary stage) or show extrahepatic dissemination within the portal tract, lymph nodes or distant visceral metastasis (Llovet et al., 2008). As recommended by Llovet and Bruix (2008), new and efficacious therapies are needed, along with new diagnostic biomarkers for early detection of liver cancer.

Liver biopsy, since its initial introduction by Klatskin as a clinical tool 100 years ago, soon became the major diagnostic test for liver disease (Afdhal & Manning, 2008). Many studies have been performed to evaluate the use of readily available laboratory biomarkers to predict significant fibrosis or cirrhosis or HCC to substantially reduce the number of performed biopsies (Attallah et al., 2007; 2009a, Ismail, 2010; Pinzani, 2010). However, biomarkers alone are not sufficient to allow definitive decisions to be made for a given patient (Halfon et al., 2008). Reproducibility studies of noninvasive markers should be performed according to professional recommendations and respecting standards previously

proposed by experts (Munteanu et al., 2008; Sanai FM, Jeeffe, 2011). However, histological examination of liver biopsy specimens is still the gold standard for evaluating the presence of liver fibrosis, pathogenesis of liver injury and assessment of anti-viral treatment. Besides establishing the diagnosis, the biopsy is often used to assess the severity of the disease in terms of both grade and stage (Patton et al., 2010).

The assessment of cellular kinetics of liver biopsies is of great importance for understanding the development of liver disease. Cellular DNA content can be measured by flow cytometry (FCM) with the aim of (1) revealing cell distribution within the major phases (G0/G1, S-phase and G2/M) of the cell cycle, (2) estimating the frequency of apoptotic cells with fractional DNA content, and/or (3) disclosing the DNA-ploidy of the measured cell population (Darzynkiewicz et al., 2010). On the other hand, Argyrophilic nucleolar organizer regions (AgNORs) proteins are a set of argyrophilic nucleolar proteins that accumulate in highly proliferating cells, whereas their expression is very low in nonproliferating cells (Fariña et al., 2011). The simple and universally applicable methods for staining fixed and non fixed liver cells will be described. Methods for staining of cell suspension from liver biopsies, and deconvolution of DNA-content-frequency histograms to estimate the percentage of cells in major phases of the cell cycle and frequency of apoptotic cells with fractional DNA content will be presented. In addition, the advantage and disadvantages of the DNA-FCM and AgNORs count in the assessment of cellular kinetics of liver biopsies in liver disease will be investigated.

2. Principles of flow cytometry

2.1 Basis of flow cytometry

Modern flow cytometers are able to analyze several thousand particles every second (analyzer flow cytometry) and can actively separate and isolate particles having specified properties; cell sorter flow cytometry (Darzynkiewicz et al., 2011). A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters . To analyze tissues, such as liver biopsies, a single-cell suspension must first be prepared using mechanical or enzymatic methods. The idea of flow cytoemtry is based on a beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically-focused stream of fluid (Cho et al., 2010).

A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals (such as propidium iodide which used for binding to DNA) found in stained cells or attached to the particle (as conjugated fluoresce antibodies used for immunophenotyping) may be excited into emitting light at a longer wavelength than the light source (de Tute, 2011). This combination of scattered and fluorescent light is picked up by the detectors, and, by analyzing fluctuations in brightness at each detector. Forward scatter (FSC) correlates with the cell size and side scatter (SSC) depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness) (Fig. 1).

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more

containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. The cell sorted machines have ability to sort cells with highly sterility and purity more than 98%, so these cells can be use for tissue culture applications (Cho et al., 2010).
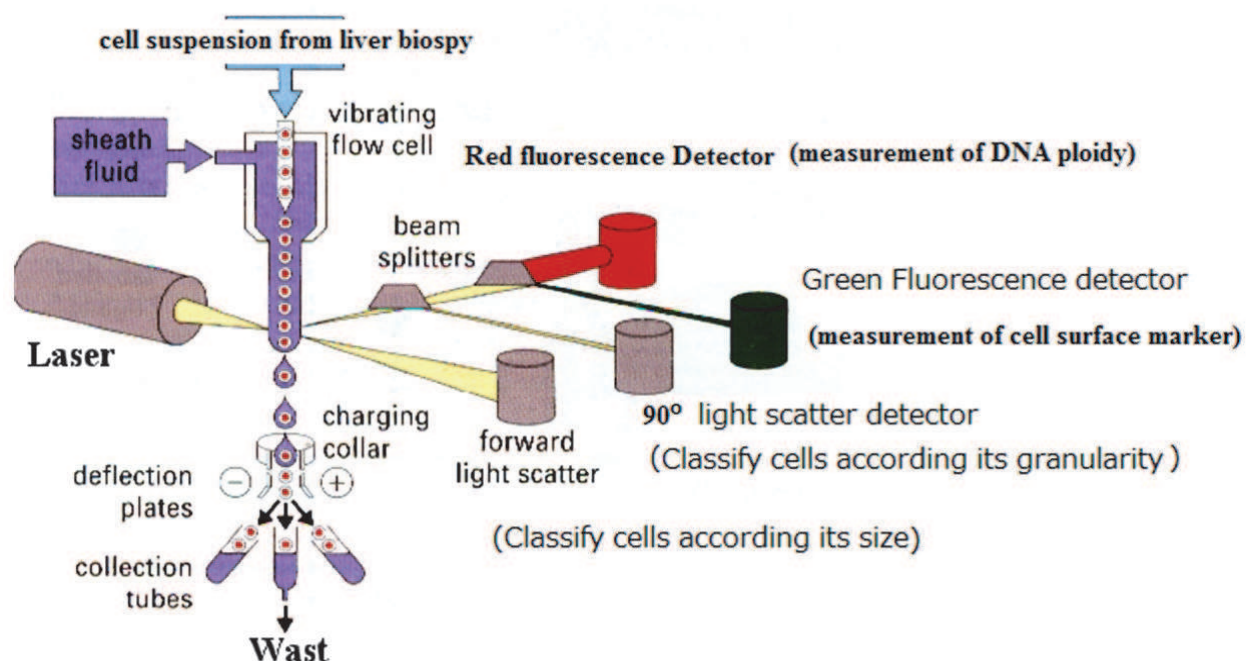

Fig. 1. Diagram of general components of flow cytometry.

2.2 DNA ploidy and cell cycle analysis
2.2.1 Cell cycle phases

The cell cycle is commonly divided into "phases" – interphase and mitosis. Interphase is further divided into three sub-phases, G1, S, and G2. In G1, cells integrate environmental and internal signals that are aimed at the "replicate"/"do not replicate" decision (Darzynkiewicz et al., 2010). The S phase is defined by the ability to synthesize genomic DNA. G2 was originally defined as the second gap between S and Mitosis, but is now known to function as a time of DNA damage repair, and likely, preparation for entering Mitosis (M phase). Mitosis has been traditionally sub-divided into stages defined by nuclear morphology – prophase, prometaphase, metaphase, anaphase A and B, and telophase. A final phase, division of the cytoplasm that overlaps telophase and is often lumped with mitosis, is cytokinesis (CK). The major cell cycle sub-phases, G1, S, G2+M can typically be identified by direct quantitative measurement of the DNA by flow cytometry based on staining of DNA with fluorescence dye such as propidium iodide (Fig. 2).
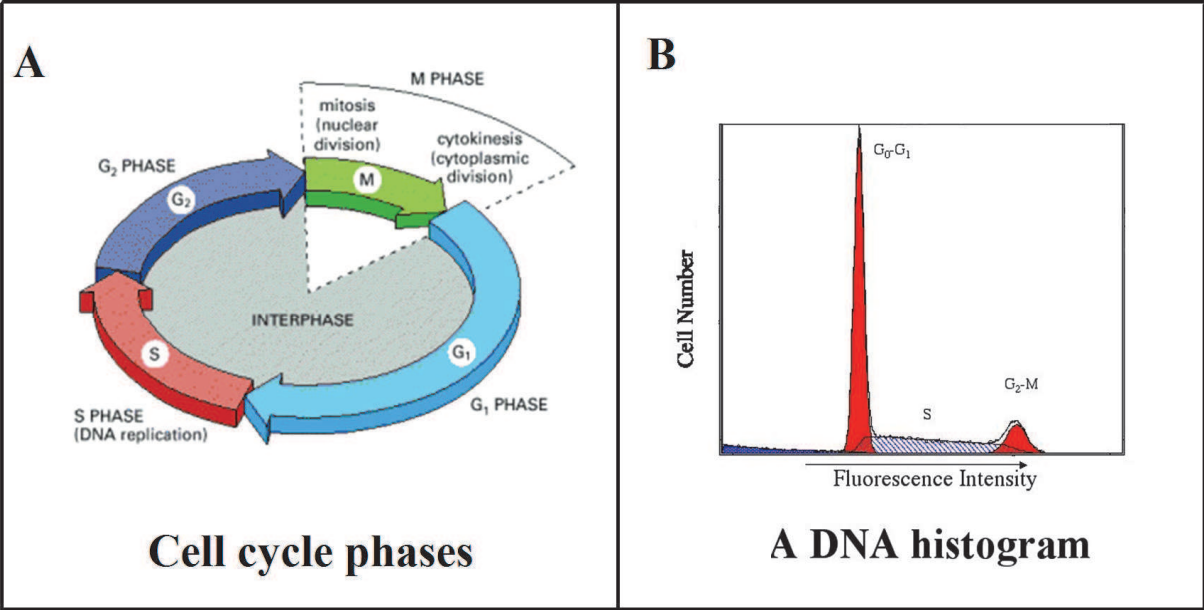
Fig. 2. The cell cycle phases (A) and diploid DNA histogram measured by flow cytometry (B).

2.2.2 DNA ploidy by flow cytometry

DNA content measurement by cytometry serves to estimate frequency of cells in particular phases ($G_{0/1}$ versus S versus G_2M) of the cell cycle as well as to assess DNA ploidy. In most situations DNA ploidy is being assessed in hematological or solid tumors; the evidence of aneuploidy by itself is a definitive marker of a presence of the tumor (Darzynkiewicz et al., 2010). Often is also considered to be a prognostic indicator of tumor progression and outcome of the treatment. To assess DNA ploidy of the tumor sample one has to compare DNA content of the $G_{0/G1}$ cells population of the presumed tumor cells with that of normal (control) cells. Towards this end most frequently the peak value of the integrated fluorescence (peak channel) of $G_{0/1}$ population of normal cells is being considered to be DI = 1.0 and DNA ploidy of the tumor cells is expressed as a ratio of the peak value (channel) of fluorescence intensity of these cells with respect to that of the normal $G_{0/1}$ cells (Darzynkiewicz et al., 2011). It is also common to express DI of the tumor as a ratio of modal rather than the peak value of fluorescence intensity representing DNA content of $G_{0/G1}$ population tumor cells to modal value of $G_{0/1}$ population of normal cells. Some authors still prefer to use the mean values of fluorescence intensity of $G_{0/1}$ population rather than the peak or modal values to obtain this ratio (Darzynkiewicz et al., 2010). In essence, when DNA measurement is done correctly and accurately, either of these approaches is expected to yield similar estimate of DI of aneuploid cells. From our experience, we can use normal lymphocytes, including lymphocytes from the same patient, as external standard control of DI = 1.0 (Fig. 3).

For comparison with the tumor it is necessary to use normal cells both as external and internal control standards. When used as external control they have to be subjected to identical processing and staining procedure and measured by cytometry under identical
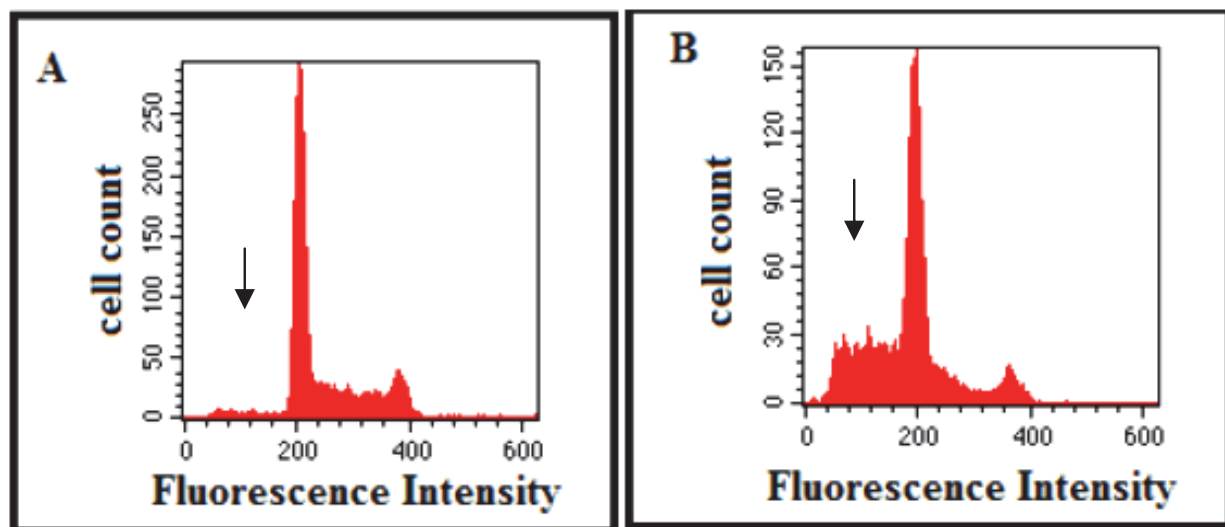
Fig. 3. Apoptotic cells (sub G0/G1 region) as measured by flow cytometry. Figure B shows high percentage of apoptotic cells (indicated by arrows) in comparison with low percentage shown in figure A.

laser and detector settings as tumor sample (Shankey and Rabinovitch, 2002). The external lymphocyte control cells should be measured prior to- and also after- measurement of tumor sample. This double- measurement of control cells allows one to detect the possible shift in fluorescence readout e.g., due to mis adjustment in instrument settings in the course of the sequential measurements. In addition to external control, normal cells should also be admixed (e.g., in 1:1 proportion) with the tumor sample cells and used then as internal control in another set of measurements (Darzynkiewicz et al., 2010). Often, normal stromal- or tumor infiltrating cells are already present in the tumor sample and they can be used as an internal control of DNA ploidy. In fact, when DNA ploidy is assessed based on measurement of nuclei isolated from paraffin blocks, the internal control provided by the presence of stromal and infiltrating normal cells that provide standard for DI = 1.0 is the only way to assess DNA ploidy of the tumor (Shankey et al.,1993). This is due to the fact that DNA stains ability after formaldehyde fixation and paraffin embedding is markedly altered making external standards useless (Darzynkiewicz et al., 2010). Two different types of reagents are necessary to ensure accuracy of DNA content analysis. Controls are used to assure proper instrument performance and to validate the DNA staining technique (Robinson et al., 2002). Fresh or fixed cells such as chicken erythrocytes (internal control or human lymphocytes (external control) have been used to determine staining consistency between samples, variations in DNA binding by different cell population can produce ambiguity in the correction definition of the diploid DNA content (Shankey et al., 1993, Darzynkiewicz et al., 2010) as shown in Fig. 4 & Fig. 5.

Fluorescent beads are useful controls to determine instrument performance and linearity because their fluorescence is consistent on a day-to day basis (Darzynkiewicz et al., 2010). These standards are used to define the amount of DNA related fluorescence that is equivalent to the amount that would be obtained from a diploid human cell.
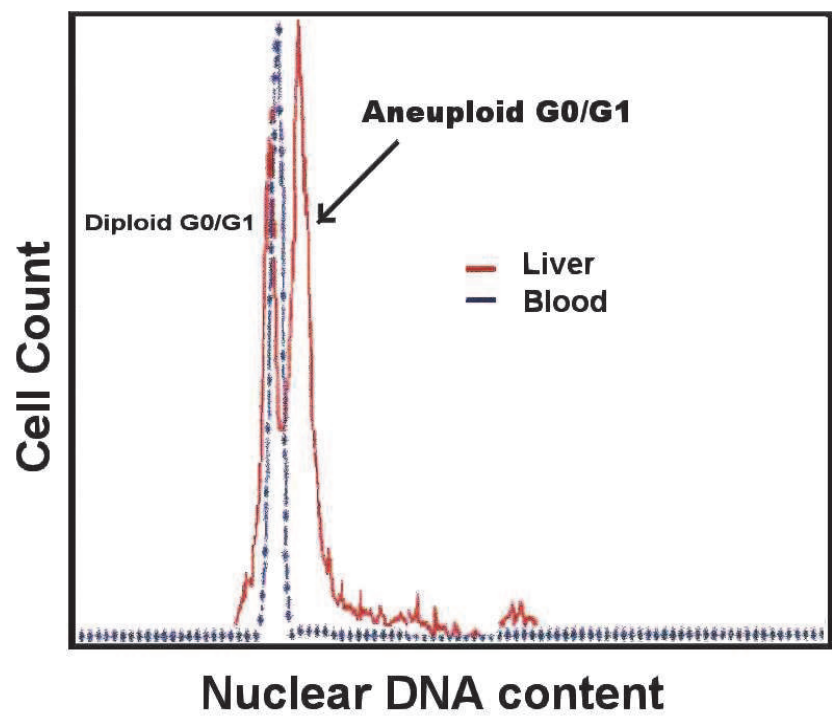
Fig. 4. Blood lymphocytes (blue) used as external control for DNA ploidy analysis by flow cytometry. DNA ploidy of liver biopsy from HCC patient (red).
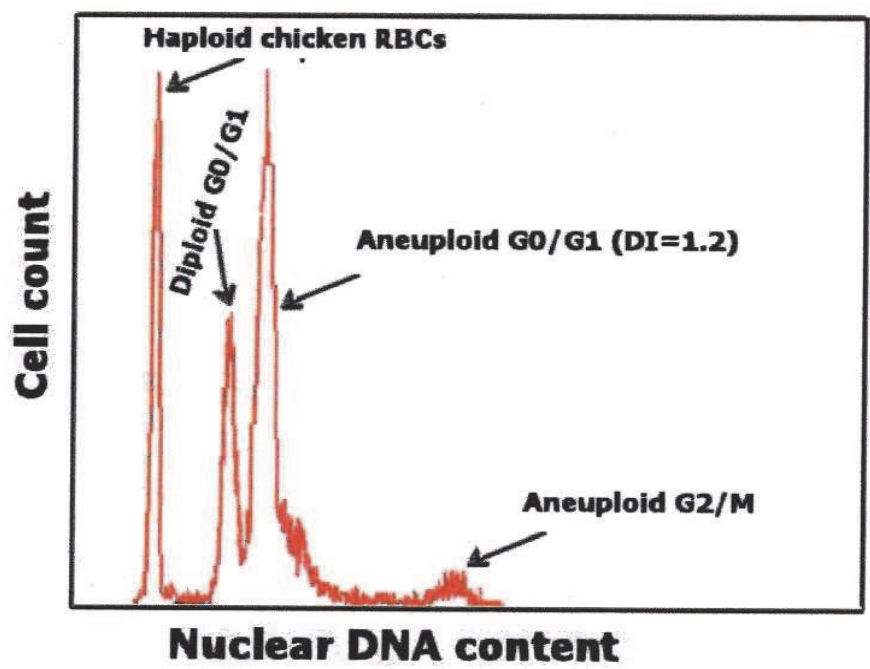

Fig. 5. Chicken red blood cells can be used as internal control during DNA ploidy analysis by flow cytoemtry.

2.2.3 Doublet discrimination

Because cells and especially fixated cells tend to stick together, cell aggregates have to be excluded from analysis through a process called doublet discrimination. This is important because a doublet of two cells in the G_0/G_1 phase has the same total amount of DNA and thus the same fluorescence intensity as a single cell in the G_2/M phase (Wersto et al., 2001). G_0/G_1 doublets would therefore create false positive results for G_2/M cells.

2.2.4 Analysis of cellular DNA content by flow cytometry

Flow cytometry analysis of ploidy and cell cycle analysis were one of the first applications of flow cytometry and this technique remains the only rapid and efficient means of making such measurements (López-Otero et al., 2010). As previously described, distribution of cells within the major phases of the cell cycle is based on differences in DNA content between the cells in pre replicative phase (G0/1) versus the cells that actually replicate DNA (S phase) versus the post replicative plus mitotic (G2 + M) phase cells (Darzynkiewicz et al., 2010). It is generally accepted that DNA content measured by cytometry (DNA ploidy) is defined as DNA index (DI) and for normal (non tumor, euploid) cells in G0/1 phase of the cell cycle DI = 1.0. Cells in G2/M phase of the cell cycle have DI = 2.0 and the S-phase normal cells are characterized by 1.0 < DI < 2.0. Different histograms DNA ploidy measured using flow cytometry based on liver biopsied from HCC and liver cirrhosis with different DNA index are shown in Fig. 6. Analysis of DNA content reveals cell ploidy, provides information on cell position in the cell cycle and also allows one to estimate frequency of apoptotic cells that are characterized by fractional DNA content (Shankey et al., 1993, Darzynkiewicz et al., 2010). Such apoptotic cells can be identified as the cells with fractional DNA content (DI < 1.0). It is often being defined as "sub-G1" cell population.
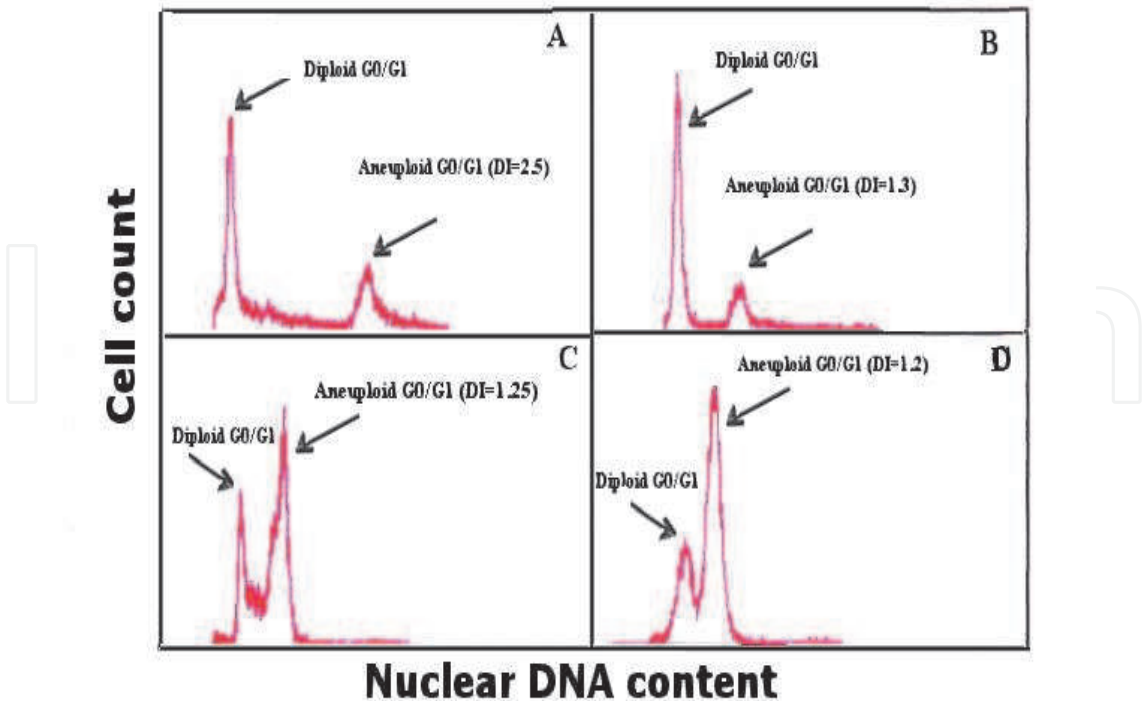

Fig. 6. Different ploidy histograms of liver biopsies from pateints with HCC. Figures A,C from tumor part and B,D from residual part (non tumor part) of HCC liver biospes.

3. Simple and universally applicable method for staining fixed and non fixed liver cells and analysis by flow cytoemtry

In cirrhotic patients with early HCC, a pre-operative liver biopsy should be performed because it has a low complication rate and it provides clinically useful information for the management of these patients (Colecchia et al., 2011). Knowledge of pre-operative tumor grade is crucial in the management of HCC because it can influence recurrence and survival after surgery. The accuracy of pre-operative needle biopsy in tumor grading has been assessed in only a few studies with conflicting results (D'Amico et al., 2009; Pawlik et al., 2007). Fine needle aspiration biopsy (FNA) is currently the preferred technique for diagnosing liver masses because of its safety and rapidity. The cytological diagnosis is usually taken as definitive, which makes it critical for patient management (Kocjan G 2010). However, in the literature it is reported that FNA of the liver has variable diagnostic sensitivity (60–100%), and that difficulties exist in diagnosing well-differentiated HCCs and in distinguishing primary from metastatic tumors (Kuo et al., 2004; Ligato et al., 2008). For this reason, additional studies that can be performed on cytological samples are desirable to aid diagnosis.

DNA analysis has diagnostic and/or prognostic significance in different human neoplasias, and can be performed on FNA samples. The following is the simple and universally applicable method for staining fixed and non fixed liver cells and analysis by flow cytoemtry. Single-cell suspensions can be prepared by mechanical dissociation of the fresh biopsy specimen in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) or phosphate buffer saline (PBS) followed by filtration through a piece of fine nylon mesh (45 μm pore-size) and centrifugation to remove debris and cell clumps. Cells were permeabilized with Triton X-100 (Sigma) (0.1 % in di-ionized H_2O followed by staining using propidium iodide (Sigma) (50 μg/ ml in PBS) as a DNA specific fluorochrome. Core liver biopsy or cell suspension can be preserved in media (sterile DMEM media or phosphate buffer saline) in freezer – 20 ^{0}C for more than one month. Also cells can fixed in absolute alcohol then be preserved at freezer – 20 ^{0}C. Flow cytometric analysis can be performed with a any flow cytometer (such as Coulter EPICS profile II Coulter Corp., Hialeah, FL), configured with a 488 nm argon ion laser. Peripheral blood lymphocytes were used as an external standard for tissue material. A total of 10000 events per sample were acquired. DNA aneuploidy was defined as any population with a distinct additional peak (s) or the presence of a tetraploid population greater than 15%. The coefficient of variation (CV) was defined as the standard deviation as a percentage of the mean DNA value of the diploid peak. Samples were excluded when CV exceeded 5%. Histograms showing only one G0/G1 peak was considered as diploid cells (normal) and those showing two distinct peaks were considered as aneuploid cells (Attallah et al., 2009b).

4. DNA-FCM in the assessment of cellular kinetics of liver biopsies in liver cirrhosis and HCC

It is scientifically and clinically important to better understand the intermediate events that predispose to neoplastic progression. Many neoplasms generate DNA-flow cytometry histograms which differ from the normal in having more than one peak with a different DNA content, the term aneuploid being used to describe these populations. Aneuploidy is believed to correlate with poor prognosis and early recurrence following surgery (Quinn

and Wright 1990; Tripathi et al., 2008). According to the multi-step theory of neoplastic progression, cancer development is associated with the evolution of a clone of cells with an acquired genomic instability and abnormal proliferation, i.e. progression to malignancy is associated with the expansion of cells with altered DNA. In our previous study, (Attallah et al., 1999b), the cellular DNA content, known as ploidy state, of cells and expressed as DI revealed that normal liver showed a diploid pattern. Abnormal DNA content was observed in 78.6% of the tumor sites of HCC, (50% were aneuploid (1:15, DI, 1:8), 28.6% were tetraploid), in agreement with Chen et al. (1991). Mise et al. (1998) found that DNA aneuploidy was observed in 31 of 80 tumors (38.8%) while Kato et al. (1998) demonstrated that, aneuploidy was seen in 45% (9 of 20) of tumors. However, 21.4% of HCC patients were in a diploid state.

Ploidy study estimates quantitative changes in chromosome complement by measuring nuclear DNA content. At least two possibilities can be considered. One is that there may be a balanced translocations chromosomal rearrangement without change in chromosomal volume, point mutations or deletions (Hayashi et al., 1988; Hiddeman et al., 1984; Raiker, et al., 1989) which cannot be identified as abnormalities by flow cytometry measurement. The other possibility is the degree of quantitative change of chromosomes. It is difficult to identify a small change, such as a defect in the short arm of a chromosome (Hayashi et al., 1988) or an abnormal or small-sized chromosome (Maletz et al, 1986; Tripathi et al., 2008). Residual liver lesions of HCC had aneuploid cell populations in 43% of cases. Aneuploidy was found in 44.5% of the liver cirrhosis patients group. The high percentage of aneuploidy in residual liver lesions of the HCC group or the cirrhotic liver group did not agree with the data of Rua et al. (1996) which demonstrated that aneuploidy is found in only 11% of cirrhotic parts of peritumoral HCC. The discrepancy is not completely clear and may be due to the method used. In the study of Rua et al., parafin-embedded tissues were used while our samples were tested fresh. In addition, dysplasia was found in 66% of cirrhotic cases and this in agreement with Anthony et al., (1973) and Roncalliet al., (2010). Aneuploidy was found in 69% of dysplastic cases. The proliferative activity (S-phase and G2/M phases), measured by flow cytometry, revealed statistically a significant difference between the liver cirrhosis group and normal liver ($P < 0.05$) and this agrees with Rua et al. (1996), who suggested that S phase fraction of cirrhotic liver parenchyma may be employed as a new additional parameter in the prognostic evaluation of HCC patients. There may be a relationship between the development of carcinoma and an increased cell proliferation, presumably due to an increased rate of random mutations (Jain et al., 2010; Tarao et al., 1994a).

The survival of patients with HCC which developed in a cirrhotic liver with a diploid DNA content and a high S-phase fraction was significantly reduced (Rua et al., 1996). Tarao et al. (1994a) demonstrated that HCC developed within a 3-year period in 64.3% of the cirrhotic patients with high DNA synthesis activity and only in 14.3% of the cirrhotic patients with low DNA synthesis activity. Ballardini et al. (1994) found higher reactivity for proliferating cell nuclear antigen in the cirrhotic liver of patients who eventually developed HCC, and therefore they suggest a differentiated follow-up of these patients. In the present study, proliferation activity of diploid tumors and residual liver lesions showed an increase but was not statistically significant ($P < 0.06$, $P< 0.08$ respectively). Patients with cirrhosis and high cell proliferation rates are at increased risk of developing cancer as reported by Baker et al. (1995) and But et al., (2008). So, the presence of liver cell dysplasia (66%) and a high proliferation rate may explain the high percentage of aneuploidy in the cirrhotic patients group and in residual liver tissues of HCC. The increase of aneuploidy in liver cirrhosis

patients and residual liver lesions of HCC demonstrated that liver cirrhosis is a premalignant lesion for HCC and that high proliferating activity and aneuploid cirrhotic patients must be followed up, as reported by Ballardini et al. (1994) and Roncalli et al., (2010). We can conclude that, DNA ploidy analysis of core liver biopsy specimens is useful in the investigation of the cell kinetics of liver cirrhosis and HCC. Dysplasia, cell proliferation and aneuploidy in liver cirrhosis may be an indication of a risk factor of liver cirrhosis for development of HCC. Also quantitative DNA content analysis using small needle liver biopsies is simple and technically convenient for flow cytometric ploidy analysis.

5. DNA ploidy and liver cell dysplasia in liver biopsies from patients with liver cirrhosis

There is controversy among pathologist when assessing the presence or absence of liver cell dysplasia in liver biopsies taken from cirrhotic patients (El-Sayed et al., 2004). Flow cytometry is a rapid cell proliferation (technique and can assess thousands of cells in a few minutes. Moreover, FCM can identify cases with high cell proliferation (high S phase and G2/M), which are very susceptible to different mutagenic agents or chemicals, which results in neoplastic transformation. FCM can therefore be considered as a better ancillary technique in the assessment of liver cell dysplasia because it is objective and not able to misinterpretation or inter-observer discordance (Attallah et al., 2009b). In our previous study (El-Sayed et al., 2004), liver cell dysplasia was found in 60 % of patients with liver cirrhosis, small cell dysplasia in 38 % and large cell dysplasia in 62 %. Our results are in agreement with finding of Roncalli et al (1989) and Lin et al., (1990). We used FCM to determine the DNA ploidy, cellular DNA content and proliferation index (PI). We found that 81.5 % of cases that were histopathologically shown to have liver cell dysplasia also had aneuploidy. In addition, aneuploidy was found in 11.1% of biopsies without liver cell dysplasia. This may be due to the higher sensitivity of FCM. These results confirm the aneuploidy nature of liver cell dysplasia (Anthony et al., 1973; Park and Roncalli et al., 1989; Roncalli et al., 2010).

The DNA content of liver cells expressed as DI was significantly higher (P<0.0001) in cases with liver cell dysplasia than cases without liver cell dysplasia, in accordance with the finding of other investigations (Henmi A, et al., 1985; Lee et al., 1997, Roncalli et al.,1989; van Dekken et al., 2005). Libbrecht et al., (2001) indicated that the presence of liver cell dysplasia in a needle liver biopsy of patients with the viral induced chronic liver disease is an independent risk factor for the development of HCC. At least two possibilities can be considered to explain the diploidy in liver cirrhosis biopsies with dysplasia. One is that there may be balanced translocations chromosomal rearrangements without change in chromosomal volume, point mutations, or deletions that con not be identified by FCM measurement (Fukushi et al., 2009; Hayashi et al., 1989; Tomioka, 2003). The other possibility may be due to the degree of quantitative change of chromosomes. It is difficult to identify a small change, such as a defect in the short arm of a chromosome. It is difficult to identify a small change, such as a defect short arm of a chromosome or an abnormal or small sized chromosome. Furthermore, the aneuploidy was found more commonly in large cell dysplasia (LCD) compared with small cell dysplasia (SCD), but no multiple aneuploid peaks (polypoidy) were found in either LCD or SCD. We showed that the cellular DNA contents were insignificant relation with activity of cirrhosis (El-Sayed et al., 2004), which is in

agreement with Lin et al., (1990). Moreover, active cirrhosis was significantly (P<0.05) associated with a higher PI (S + G2M phases) than diploid cases with inactive cirrhosis and my due to liver response to cell injury (Pahlavan et al., 2006; Schaffner 1991). The value of detecting these cellular DNA changes in liver cirrhosis by FCM is of prognostic value in regards to early detection of preneoplastic changes in liver cirrhosis were found to be at increased risk of developing HCC (Clemente et al., 2007; Ruà et al., 1996; Tarao et al., 1994b). The presence of an aneuploid DNA pattern had been reported by Hoso and Nakanuma (1991), to a precancerous change in cases with atypical adenomatous hyperplasia, of the liver. Yet, the presence of liver cell dysplasia, although not specific as premalignant condition, could be considered as a serious hand mark in cases of liver cirrhosis. In our previous study, DNA aneuploidy was indentified in 46.7 % of patients with liver cirrhosis (El-Sayed et al., 2004). However, Thomas et al., (1992) reported a lower incidence (25 %) of DNA aneuploidy in patients with liver cirrhosis. This difference may be due to the use of archival material of paraffin embedded blocks. Also serum albumin levels was significantly lower (P<0.008) in patients with liver cell dysplasia than patients without dysplasia. Moreover, a significant negative correlation (P<0.05) was found between the serum albumin and the percentage of aneuploid cells. This may be a attributed to poor synthesis of albumin by these dysplastic hepatic cells as a result of change in the nuclear DNA contents of these aneuploid cells. So we can conclude that liver cell dysplasia is associated with aneuploidy, as measured by flow cytometry, may carry the risk for progression to HCC and liver cirrhotic patients with aneuploidy must be followed up.

6. Advantage and disadvantages of the DNA-FCM count in the assessment of cellular kinetics of liver biopsies in liver disease

Measurement of DNA content of individual cells by using flow cytometry offers a number of advantages over cytogenetic analysis (Nunez et al., 2004; Ritter et al., 1994). Unlike cytogenetic analysis, in which 20 to 40 cells are routinely analyzed, cytometric analysis can measure ten thousands of cells or nuclei. One of the most advantages of cytometric DNA content analysis is that it does not rely on metaphase cells, and cell in all phases of the cell cycle are generally included in the analysis. The critical limitation of cytometric analysis is the significantly lower resolution compared with karotyping. Flow cytometric DNA content analysis con not provide information on chromosome structure and is thus incapable of detecting balance chromosomal translocation (Dundar et al., 2010; Shankey et al., 1993). The limitation imposed on cytometric analysis even using high precision instruments and the most stiometric DNA binding fluorescence dye; DAPI (4, 6 diamidino 2-phenylindole), prevent cytometric from detecting chromosomal gains or losses involving more than about 5 % of the total DNA. In practical terms, this implies that the smallest change that can be detected theoretically is the gain or loss of equivalent of one large (e.g. A-group) chromosome (Shankey et al., 1993).

7. Nucleolar organizer regions

Nucleolar organizer regions (NORs) are situated within the nucleolus of a cell that are of central importance in the transcription of DNA to ribosomal RNA and hence in the ultimate assembly of proteins (Howat et al., 1989; Schwint et al., 1996; Uno et al., 1998). NORs are located, one each on the short arms of the five acrocentric chromosomes namely

13, 14, 15, 21, and 22 as secondary constrictions close to the centromere (Howell, 1982; Vuhahula et al., 1995). The number of NOR-bearing chromosomes are seen in the karyotype (Landini et al., 1990). NORs could be demonstrated in histological sections by means of the argyrophilia of their associated proteins using the structures and simple silver staining method and thus demonstrated as termed AgNORs (Crocker, 1995; Crocker & Skilbeck 1987; Trere`, 2000).

AgNOR proteins are a set of argyrophilic nucleolar proteins that accumulate in highly proliferating cells, whereas their expression is very low in nonproliferating cells. Some of these proteins remain associated with the NORs during mitosis. In situ, the expression of AgNOR proteins is measured globally by quantification of the level of silver staining using morphometry and image analysis (Sirri et al., 2000). The distribution of AgNORs and their quantities have been associated with proliferative activity and ploidy in different neoplastic and preneoplastic conditions. Several studies investigated the correlation of the number of detectable AgNORs with the degree of nucleolar dispersion or disaggregation, which is related directly to cell proliferation and the NOR-bearing chromosomes (Arora et al., 2002; Giri et al., 1989; Underwood & Giri, 1988).

7.1 Staining of nucleolar organizer regions

In our laboratories, liver biopsy samples were taken from the most representative area of the diseased tissue. The biopsy specimens were then fixed in 10% buffered formalin and subsequently processed to prepare paraffin blocks. Two separate sets of sections of 4 μm thickness were obtained from each block. One set stained by the hematoxylin and eosin (H&E) technique for routine histological evaluation and confirmation of diagnosis. Another set of sections were stained for localization and quantification of AgNORs according to the method described by Lindner et al., (1993). The sections were deparaffinized in xylene, and hydrated through 100% and 95% ethanol to water. Reduction of sections was done with 1% potassium iodide for 60 min. Silver staining solution was prepared by dissolving 2% gelatin in 1% formic acid at room temperature and filtered through syringe filter (Sigma Chemical Co., St. Louis, MO, USA). One part of the solution was mixed with two parts of 50% silver nitrate immediately before use. Staining was done in dark at room temperature for 30 min, then sections were immersed in 5% sodium thiosulphate for 5 min. Sections were washed in water, dehydrated in 95% and 100% ethanol, and mounted in permanent mounting media (Attallah et al., 2009b).

7.2 Counting of silver stained nucleolar organizer regions (AgNORs)

AgNORs appear as brown black dots inside the nucleus using silver stain. Counting of AgNORs was done without any knowledge of the histological or the clinical data. The slides were examined with an oil immersion objective at 1000 magnification. The examined fields were selected randomly. AgNORs were counted in 100 nuclei, which were randomly selected. The absolute number of AgNORs within each nucleus was counted and the mean number of AgNORs per nucleus (mAgNORs) was determined for each case (fig. 7). Background should be pale yellow and clear, the nucleus yellow and/or brown, and AgNOR sites intra-nucleolar black dots.

Most studies have shown that high AgNORs counts can reflect a tumor's degree of malignancy, differentiate among reactive, benign, and malignant conditions (Derenzini & Ploton 1991; Khan et al., 2006; Kummoona et al., 2008; Raikhlin et al., 2006). Crocher &

McGovern (1988) applied the AgNORs method to normal, cirrhotic, and carcinomatous livers and recognized a significant difference in their AgNORs scores. Study of Jain et al. (1998) indicated that both quantitative and qualitative analysis of AgNORs may be useful as an adjunct to routine hematoxylin and eosin stain to distinguish cirrhosis from HCC especially when the latter is well differentiated. Also, AgNORs count in the hyperplastic foci was significantly higher than that in the controls (Wakasa et al., 1998). Jagan et al. (2008) investigated the antiproliferative effect of gallic acid during diethylnitrosamine (DEN)-induced HCC in male Wistar albino rats. They used levels of AgNORs as a tumor marker, which can be an indicator of tumor response to therapy.
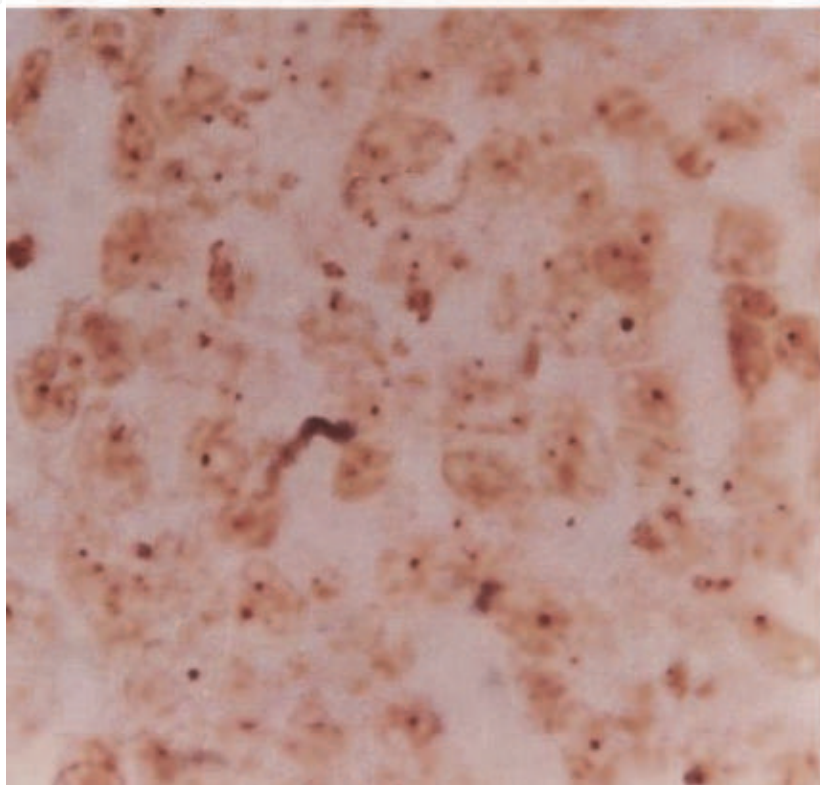

Fig. 7. The AgNORs staining in liver of patient with HCC. The findings were viewed following staining under the light microscope using 100X oil immersion lens and cedar wood oil. Nucleolar organizer regions appear as brown black dots inside the nucleus using silver stain (AgNORs count).

7.3 AgNOR count reflects DNA ploidy or proliferative activity

Several attempts have been made to determine whether mean AgNOR count reflects ploidy or proliferative activity. However, several studies have correlated the mean AgNOR count with Ki-67, bromodeoxyuridine (BrdU)-labeling indexes (Munakataet al., 1994; Trere et al., 1991), and proliferating cell nuclear antigen (PCNA) in mucosa-associated lymphoid tissues and gastrointestinal stromal tumors (Yu et al., 1992). Mikow et al. (1993) indicated that mean AgNOR reflects the proliferative activity rather than ploidy. Another study failed to show a relationship between AgNORs count and Ki-67 and BrdU-labeling indexes (Nyska, 1995), or between diploid and aneuploid tumors (Nagao et al., 1995). This is in a disagreement with a study of mean AgNOR in

trophoblastic disease, which indicated that mean AgNOR is a reflection of ploidy and proliferative activities (Maier et al., 1990). However, in contrast to immunohistochemical methods merely determining the growth fraction of the cells by Ki-67 and proliferating cell nuclear antigen (PCNA) analysis AgNORs apparently reflect time-related proliferation rates and ploidy. Thus, AgNOR analysis may provide further insights concerning the biological behavior of the cell and is simple, inexpensive, and reliable method of evaluating the proliferative activity and ploidy of the cell (Meng et al., 1996).

Celikel (1995) found a correlation between mean AgNOR and ploidy and proliferation activity of urinary bladder tumor as measured by DNA flow cytometry. Also, Borzio et al. (1998) demonstrated that a high hepatocyte proliferation rate detected by AgNORs is a major risk factor for HCC development in the cirrhotic liver. Therefore, they indicated that the evaluation of the hepatocyte proliferation rate is very important to identify patients requiring stricter follow-up program for early diagnosis of HCC. High AgNOR count in cirrhotic liver as a marker of regenerative capacity has been associated with a significantly increased incidence in development of HCC in chronic liver disease (Derenzini et al., 1993). However, in liver cirrhosis, dysplasia had a significant relationship with ploidy and AgNORs. So, the incidence of high AgNORs count, dysplasia, and presence of aneuploidy by DNA flow cytometry suggest that liver cirrhosis may be considered as a more serious condition in the evolution of HCC.

Recently, we compared the results of DNA flow cytometry and AgNORs with the histopathological data. It was found that the lowest mean AgNOR count per nucleus was found in the normal liver (1.3± 0.9) and the highest count was (3.89± 0.827) in tumor lesion of HCC (Attallah et al., 2009b). A gradual increase in the AgNOR count per nucleus was noted as disease progressed from normal liver through liver cirrhosis to HCC tumor lesions. Analysis of AgNOR counts showed a significant difference between tumor and residual liver tissues of HCC or normal liver (P < 0.001) and this is in agreement with several studies (Shemizu et al., 1995; Siddiqui et al., 1999; Politi et al., 2004). However, Chen et al. (2003) revealed that AgNOR area and AgNOR area-count ratio are the most valuable features for differential diagnosis of normal, preneoplastic, and cancer cells. Spolidorio et al. (2002) found that the morphometric results were statistically different for normal mucosa, dysplasia, and microinvasive oral carcinoma and they concluded that an increase of NOR activity follows the disease progression and may reflect the degree of cellular proliferation and malignancy. Siddiqui et al. (1999) revealed a gradual increase in mean AgNOR counts from normal liver through cirrhosis to HCC. The difference in NOR counts was significant in these three groups. The hepatocellular carcinomas were graded according to the Edmondson-Steiner histological grading system. Grade I hepatocellular carcinomas show AgNOR counts ranging between 5 and 6/cell, a score that is much higher than in the normal liver, where it ranges between 1.2 and 2.0/cell. This technique can be used to assess the lesions where the distinction between normal liver and grade I HCC is difficult with the use of routine methods, on the light of the hypothesis of a relationship between the development of carcinoma and an increased cell proliferation, presumably by an increase of random mutations (Jain et al., 2010; Tarao et al., 1994a). So, quantification of AgNOR is considered to have putative relationship with ploidy in HCC. Our study presents complementary methods to histopathology, which are valuable to pathologists, when they have difficulty in diagnosis of early stages of HCC on the basis of liver biopsy. Finally, both AgNORs count and DNA ploidy analysis are useful and valuable indicators of cellular kinetics in HCC.

8. Conclusion remarks and future research

The assessment of cellular kinetics of liver biopsies is of great importance for understanding the development and diagnosis of liver disease. Flow cytometric analyses of ploidy and cell cycle were one of the first applications of flow cytometry and this technique remains the only rapid and efficient means of making such measurement particularly for core liver biopsies. AgNOR proteins are a set of argyrophilic nucleolar proteins that accumulate in highly proliferating cells, whereas their expression is very low in nonproliferating cells. The AgNOR tissue marker can be used as a routine complementary histopathologic study and DNA ploidy by flow cytometry, since the variations in its number and distribution indicate existence of cell alterations in a given lesion and the use of this technique is easy and inexpensive. Diagnosis of HCC using both DNA ploidy and mean AgNORs are useful in the detection of cellular and structural abnormalities. However, further research are required to confirm the relationship between viral infections such as HCV and cancer, cell proliferation and apoptosis based on liver biopsies from patients cirrhosis and HCC.
The detection of viral antigens based on specific monoclonal antibodies and/or the detection of viral hepatitis nucleic acids such as HCV RNA and HBV DNA in the liver biopsies and its relation to cell cycle analyses measured by flow cytometry will be the future research in this field.

9. Acknowledgment

10. References

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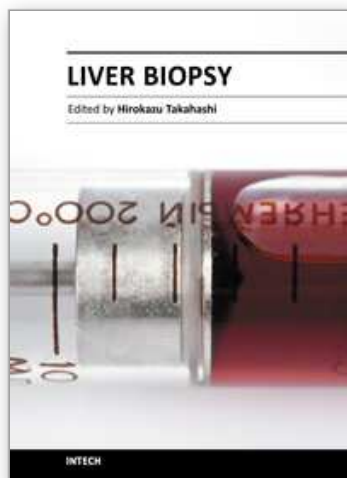
Liver Biopsy

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Liver biopsy is recommended as the gold standard method to determine diagnosis, fibrosis staging, prognosis and therapeutic indications in patients with chronic liver disease. However, liver biopsy is an invasive procedure with a risk of complications which can be serious. This book provides the management of the complications in liver biopsy. Additionally, this book provides also the references for the new technology of liver biopsy including the non-invasive elastography, imaging methods and blood panels which could be the alternatives to liver biopsy. The non-invasive methods, especially the elastography, which is the new procedure in hot topics, which were frequently reported in these years. In this book, the professionals of elastography show the mechanism, availability and how to use this technology in a clinical field of elastography. The comprehension of elastography could be a great help for better dealing and for understanding of liver biopsy.

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