Hepatic mRNA expression of histone (H3): an early predictor of tumorgenic changes in chronic hepatitis C

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Abstract

Introduction: Infection with hepatitis C virus (HCV) persists in most infected individuals and can lead to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Histones are numerous DNA-binding proteins that control the level of DNA condensation and play a critical role in the dynamic changes that occur during the cell cycle. The aim of this study is to assess the changes of histone 3 (H3) mRNA cell cycle gene expression as early predictor of tumorgenic changes in chronic hepatitis C (CHC).
Material and methods: The study was conducted on liver biopsies from fifty patients with CHC and HCC. Ten normal liver biopsies taken during surgical cholecystectomy served as controls. Histone H3 mRNA expression was evaluated in formalin- fixed and paraffin-embedded liver tissue sections by *in situ* hybridization.
Results: Out of the fifty cases, 20 cases were diagnosed as CHC, 10 cases with HCV-induced liver cirrhosis and 20 cases as HCC on top of HCV. Hepatic expression of histone H3 was higher in CHC or HCC patients than in control group. The cytoplasmic expression of H3 was higher in HCC and cirrhotic liver disease, while the nuclear expression of H3 was more prominent in CHC especially lower scores of necro-inflammatory activity and fibrosis.
Conclusions: It could be concluded that alterations of hepatic expression of histone H3 mRNA quantitatively (increased expression) and qualitatively (nuclear-cytoplasmic shift) may be used for early prediction of tumorgenic changes in CHC.

Key words: histone (H3), *in-situ* hybridization, chronic hepatitis C, hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is one of the major health problems throughout the world. It is the fifth most common cancer in the world, accounting for an estimated 500,000 deaths annually [1, 2]. Although much is known about both the cellular changes that lead to HCC and the etiological agents responsible for the majority of HCC cases (Hepatitis B virus, Hepatitis C virus, alcohol), the molecular pathogenesis of HCC is not well understood [3, 4].

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Chronic infection with hepatitis C virus (HCV) is a well-established risk factor for the development of HCC. Chronic infection with HCV appears to cause HCC as a result of chronic or recurrent hepato-cellular necrosis and hepatic inflammation and regeneration [4]. The HCV itself has direct carcinogenic potential. The HCV core protein has been implicated as being possibly carcinogenic as a result of its effect on normal growth, apoptosis, repair and cellular signaling pathways [5].

Cancer is recognized as a genetic disorder at the cellular level that involves mutation of a small number of genes. Many of these genes normally act to suppress or stimulate progression through the cell cycle, and loss or inactivation of these genes causes uncontrolled cell division and tumor formation [6].

Molecular genetics are concerned with the inter-relation between the information macromolecules DNA (deoxy-ribonucleic acid) and RNA (ribonucleic acid) and how these macromolecules are used to synthesize polypeptides, the basic component of all proteins. In human cells, genetic information is stored in DNA molecules [7]. A variety of nucleotide sequences in DNA and RNA can serve as markers for the molecular genetic diagnosis of cancers. Most of these markers directly or indirectly represent alterations in DNA that distinguish malignant cells. These markers may be related to the cause of cancer (e.g mutations in oncogenes or tumor suppressor genes) or to modifications in nucleic acid sequences that are acquired during the progression of the tumor [8].

Cells protect their DNA by organizing it as a higher order nucleoprotein complex termed chromatin in which the basic unit is the nucleo-some. Each nucleosome is composed of an octamer of core histones (two each of H2A, H2B, H3 and H4), around which two super-helical turns (80 base pairs) of DNA are wrapped [9, 10]. Histones are the numerous DNA-binding proteins that control the level of DNA condensation, and post-translation modification of histone tails plays a critical role in the dynamic condensation/decomposition that occurs during the cell cycle [11, 12].

Histones have an unusual amino acid composition in that they are extremely rich in the positively charged amino acids lysine and arginine. This property enables them to bind to the negatively charged phosphates of the DNA so they have an important role in the condensation of the DNA in chromatin [11]. Elevated levels of histone H3 and phosphorylated H3 may be responsible for the less condensed chromatin structure and aberrant gene expression observed in the oncogene-transformed cells [13, 14]. Aberrant acetylation or deacetylation of histone lead to many human disorders including cancer. The various groups of histone acetyl transferases and deacetylase were regarded to be involved in cancer progression and human diseases [15-18].

Determining the proliferative activity of tumor cells is very important for diagnostic purposes and subsequent treatment decisions. Evaluation in tissue sections is generally performed by quantitation of mitotic figures, PCNA or Ki-67 immunohistochemical labeling, silver argyrophilic nuclear organizer region staining (AGNORs), thymidine (or BrdU) labeling and flow cytometry [19]. Each of these methods has its own merits and limitations [20].

Synthesis of histones was associated with DNA replication but these synthetic rates might be different in tumor cells [11, 21]. All histones were not detected in human HCC, only H3 was detected on western blot, while H1, H2A, H2B, H4 were not detected in human HCC [11].

In situ hybridization (ISH) for some histone mRNA species, including histone 3 (H3) has been introduced as an extremely accurate technique for assessment of S-phase cell proliferation indices [22]. Thus the detection of H3-mRNA-expressing cells can be used to evaluate the proportion of cells in the S-phase, because H3-mRNAs are rapidly degraded after DNA synthesis is complete [23].

The present study employing histone H3-mRNA *in-situ* hybridization was undertaken to assess cell kinetics and clarify the relationship between proliferation activity and the H3-mRNA expression in chronic hepatitis C and subsequent hepato-cellular carcinoma.

Material and methods

Patients and controls

This study was conducted on 50 chronic liver disease (CLD) patients with age range for 20 to 63 years (32 males and 18 females) admitted to the inpatient section of Hepato-Gastroenterology department, Theodor Bilharz Research Institute (TBRI). All patients were subjected to thorough clinical examination and laboratory investigations including urine and stool analysis, liver function tests and serological diagnosis of schistosomiasis and hepatitis (B and C). Patients were further subjected for abdominal ultrasound (Hitachi EuB-515A) and liver biopsy specimens were obtained from hepatitis patients by percutaneous needle using the Menghini-technique and from HCC patients by ultrasound-guided needle biopsy for histopathological and *in-situ* hybridization studies. All biopsied patients had prothrombin time ≥ 60% to avoid bleeding. Serum samples from all studied subjects were collected, aliquoted and kept frozen at −70°C till further use. Patients who had any history of alcohol intake or other causes of chronic

liver disease than CHC and its sequelae; cirrhosis and/or HCC, were excluded from the study.

The control group included 10 wedge liver biopsy specimens taken during surgical cholecystectomy from ten healthy subjects, well matched for age and sex. They all had clinical, biochemical, serological, ultrasonographic and histological findings within the normal range. Our study followed the tenets of the Declaration of Helsinki [24].

Laboratory tests

Liver function tests including albumin, aspartate aminotransferase (AST) and alanine amino-tranferase (ALT) were carried out using commercially available kits. Hepatitis B markers including hepatitis B surface antigen and anti-HBs antibodies, total and IgM class antibodies against hepatitis B core antigen, HBe antigen and anti-HBe antibodies were tested using enzyme immunoassay kits (Abbott laboratories, North Chicago, IL). Circulating anti-HCV antibodies were detected using a third generation enzyme immunoassay kit (Murex anti-HCV, Version III, Murex Diagnostics, Dart ford, England). Serum levels of HCV RNA were detected by nested RT-PCR according to Saber *et al.* [25]. Serum α-fetoprotein (α-FP) was measured using enzyme immunoassay kit (Euro genetics N-V Belgium).

Histopathological procedures

Liver specimens were fixed in 10% buffered formalin and processed into paraffin blocks. Routine hematoxylin-eosin and Masson trichrome stained sections were prepared for all cases. As regard CHC, the degree of hepatic necro-inflammation and the stage of fibrosis were scored according to the METAVIR system [26]. Grade 1 inflammation as well as the first and second stages of fibrosis were considered low score, while, grades 2 and 3 inflammation as well as the third and fourth stages of fibrosis were considered high. Biopsies of HCC were graded according to the histological differentiation of Ishak [27].

According to the above mentioned investigations, patients were classified into three groups: group I – 30 patients diagnosed as CHC, group II – 20 patients diagnosed as HCC on top of HCV-infection, group III – 10 normal controls.

In situ hybridization (ISH)

We performed H3 mRNA-ISH on hepatic tissues using the method described by Maeyama *et al.* [28]. All reagents, including histone H3 probe, were obtained from Dako (Glostrup, Denmark). The H3 probe is a 550-base single-stranded, antisense, fluorescein-labeled DNA fragment that hybridizes to the entire coding region of the human histone H3 mRNA. It was used for *in situ* hybridization in

the hybridization buffer supplied by the manufacturer. Briefly, for formalin fixed specimens, deparaffinized, rehydrated slides were treated with protinase K for 5 min and rinsed in distilled water several times. Sections were immersed in pre-warmed Target Retrieval solution for 20-40 min at 95°C. Fluorescein-labelled H3 probe was applied to the sections and incubated overnight at 55°C. The slides were then washed in pre-warmed stringent wash buffer 1 : 50 (Dako) at 55°C for 15 min and rinsed in Tris-buffered saline (TBS). Sections were then incubated with alkaline phosphatase-conjugated anti-fluorescein antibodies at room temperature for 20 min. Development was performed in BCIP/NBT substrate for 2 h. The slides were then dehydrated and coverslipped. Negative control was treated identically except that the H3 probe was replaced by TBS.

Assessment of H3-mRNA-ISH staining

Randomly selected fields of the stained slides were evaluated at high power for H3 labeling. A minimum of 400 cells from three different high powered (40×) fields were examined for each slide. We counted the numbers of positive and negative cells in each hepatic tissue. Scores were then obtained by calculating the percentage of cells showing positive nuclear and/or cytoplasmic staining. The H3-mRNA-ISH was evaluated independently by two observers (T.A and M.M).

Statistical analysis

The data are presented as mean ± standard error of mean (X ± SEM). The means of the different groups were compared globally using the analysis of variance ANOVA test, on SPSS software version 9. The data were considered significant if *p* values were less than 0.05.

Results

Fifty patients, 32 males and 18 females (mean age 20 to 63 years), with circulating anti-HCV antibodies and no serological evidence of co-infection with hepatitis B virus were selected for study. All patients were sero-positive for HCV RNA as detected by nested RT-PCR.

Tables I and II showed that patients with CHC accompanied with cirrhosis or HCC were more symptomatic with more evident signs of CLD than those without cirrhosis.

There was a significant increase in liver enzymes (ALT and AST) in HCC group (*p* < 0.01) compared to CHC group with a significant decrease in serum albumin level (*p* < 0.001) in HCC than CHC group. As regard the HCV markers, both hepatitis C and HCC groups showed seropositivity for anti-HCV antibodies. However, the quantitation of the

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Table I. Characteristic features of the studied patients

Parameter	CHC		HCC
	Without cirrhosis	With cirrhosis	
Number of patients	21	9	20
Age	35.80 ±9.54	49.55 ±9.05	50.00 ±7.90
Sex ♂/♀	14/7	6/3	12/8
Fatigue	15 (71.4%)	7 (77.7%)	20 (100%)
Myalgia	10 (47.6%)	8 (88.8%)	12 (60%)
Arthralgia	8 (38%)	4 (44.4%)	6 (30%)
Right hypochondrial pain	18 (85.7%)	6 (66.6%)	16 (80%)
Pruritis	2 (9.5%)	1 (11.1%)	9 (45%)
Jaundice	1 (4.76%)	4 (44.4%)	8 (40%)
Palmar erythema	0	9 (100%)	20 (100%)
Spider naevi	0	8 (88.8%)	16 (20%)
Liver			
Normal size	4 (19%)	1 (11.1%)	6 (30%)
Enlarged	17 (80.9%)	2 (22.2%)	5 (25%)
Shrunken	0	6 (66.6%)	10 (50%)
Splenomegaly	3 (14.3%)	6 (66.6%)	16 (80%)
Ascites	0	0	0

CHC – chronic hepatitis C, HCC – hepatocellular carcinoma

Table II. Abdominal ultrasonography of the studied patients

	CHC		HCC
	Without cirrhosis (n = 21)	With cirrhosis (n = 9)	(n = 20)
Liver size			
Normal	3 (14.3%)	1 (11.1%)	4 (25%)
Enlarged	18 (85.7%)	2 (22.2%)	5 (25%)
Shrunken	0	6 (66.6%)	11 (55%)
Texture			
Homogenous	4 (19%)	0	0
Bright	17 (80.9%)	1 (11.1%)	0
Coarse	0	8 (88.8%)	20 (100%)
Focal lesion			
Size < 3 cm	0	0	5 (25%)
3-5 cm	0	0	10 (50%)
> 5 cm	0	0	5 (25%)
Splenomegaly	4 (19%)	8 (88.8%)	18 (90%)

CHC – chronic hepatitis C, HCC – hepatocellular carcinoma

hepatitis C viral genome by PCR test showed a highly significant increase of the HCV particles in sera of HCC compared with those of CHC group ($p < 0.01$) (Table III).

Nuclear expression of histone H3 was found to be statistically higher in cases of CHC than in HCC, while cytoplasmic expression showed the reverse ($p < 0.05$) (Table IV). It was found that with increasing grade of necro-inflammatory activity and stages of fibrosis in CHC cases, the nuclear expression of H3 was decreased while cytoplasmic expression showed increasing values compared with lower scores but with no statistical difference (Table V, Figures 1A, 1B). As regards the different grades of HCC, cytoplasmic expression of H3 was found to be statistically more in high grade than in low grade ($p < 0.01$) (Table VI, Figures 1C, 1D).

Discussion

The expression of histone genes is a fundamental step constituting the process of cell proliferation [29]. Evaluation of cell proliferative activity in tissue sections can be achieved by many different methods. *In vivo* labeling of DNA by nucleotide analogues such as [3H] thymidine or BrdU has proven useful for identifying S-phase cells in animal experiments, but this "gold standard" is not generally suitable for human studies, especially when repeated samplings are required from the same individual [30, 31]. However there are problems of epitope masking or loss, and detectable antigen levels can vary among different cell types, leading to spurious differences in the calculated proliferation rate [32, 33]. Proliferation-related functions can be regulated not only by protein levels but also by conformational changes not detectable by immunohistochemistry [34]. An alternative approach is the expression of the histone H3 gene that has shown to be tightly coupled to DNA synthesis in all cell types examined (normal and malignant) [35]. The non isotopic histone mRNA *in situ* hybridization was proved to be a useful method for estimating cell proliferation in clinical pathology [36].

In this study, nuclear expression of H3-mRNA was decreased with increasing grade of hepatitis activity while cytoplasmic expression showed increasing values with increased grades of hepatitis and stages of fibrosis. This could be explained by accumulation of H3 in the hepatocytic nuclei during early hepatic insult. With progression of the disease, liberation of an already formed H3 took place from the nucleus into the cytoplasm of the hepatocytes.

S-phase markers (e.g. histone mRNA) label only those cells that have passed the G1/S boundary. The expression of the histone genes (H2A, H2b, H3, H4) is closely connected to the replicative stages of the cell cycle [37]. When DNA synthesis is

Table III. Values of sero-diagnostic investigations in different studied groups

| Groups | Liver function tests | | | HCV markers | | αFP [u/ml] X±SEM |
	ALT [u/l] X ± SEM	AST [u/l] X ± SEM	Albumin [g/dl] X ± SEM	HCV-Ab	HCV-PCR [genome/ml] X ± SEM	
Control ($n = 10$)	40.0 ±2.5	45.0 ±2.1	4.5 ±1.3	–	–	8 ±1.7
CHC ($n = 30$)	47.7 ±9.77	44.4 ±7.96	4.2 ±0.13	+	245.0 ±25.15	10 ±2.1
HCC ($n = 20$)	88.9 ±8.09[a]	127.2 ±16.35[a]	2.8 ±0.14[b]	+	502.0 ±27.91[a]	2000 ±1.9[b]

[a]$p < 0.01$ vs. chronic hepatitis C (CHC), [b]$p < 0.001$ vs. CHC, ALT – alanine aminotranferase, AST – aspartate aminotransferase, α-FP – α-fetoprotein

Table IV. Expression of histone H3 in different studied groups

Groups	No of patients	Nuclear expression Mean ± SEM	Cytoplasmic expression Mean ± SEM
Control	10	11.00 ±5.66	4.20 ±4.91
Chronic hepatitis C	30	70.26 ±20.91[a]	65.01 ±25.44[a]
Hepatocellur carcinoma	20	53.33 ±28.28[ab]	84.44 ±18.10[ab]

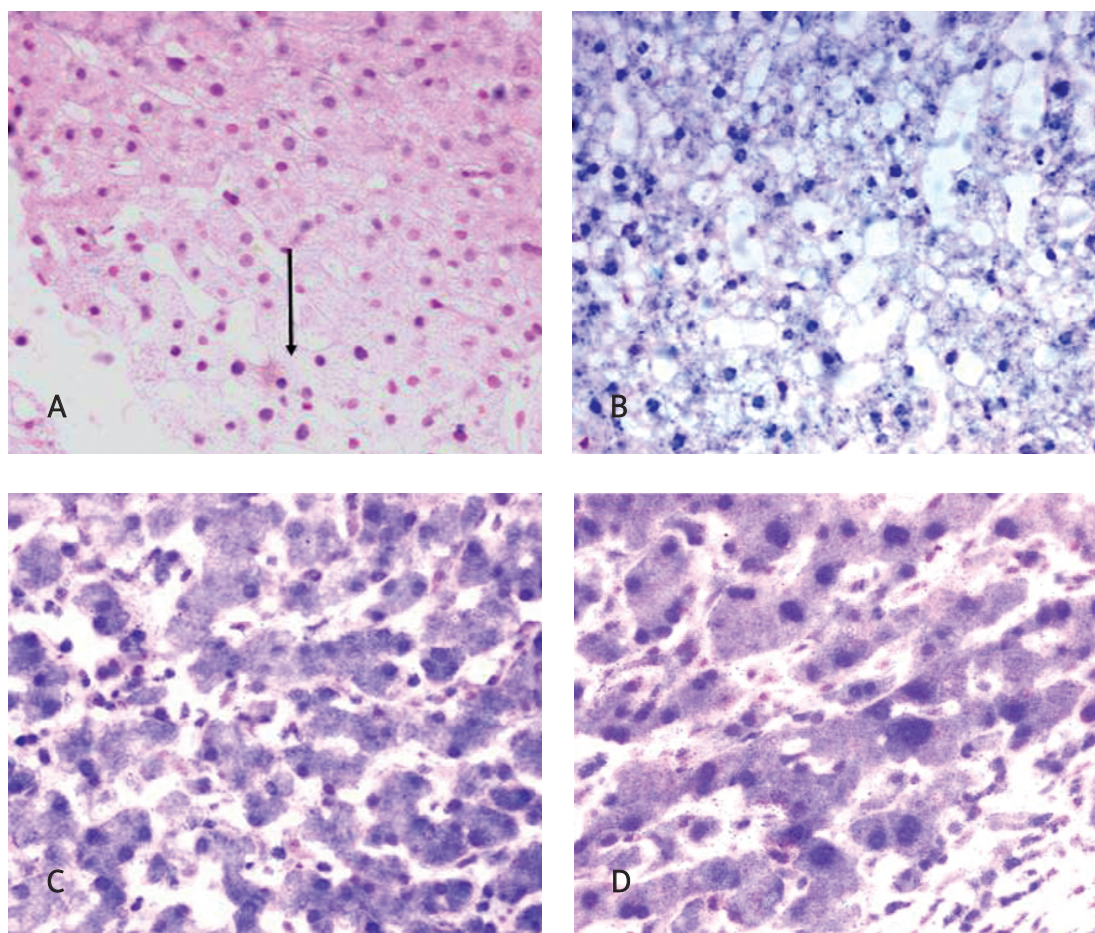[a]$p < 0.01$ vs. control group, [b]$p < 0.05$ vs. chronic hepatitis C

Figure 1. Photomicrography of Histone H3 transcripts in liver sections showing: focal nuclear expression of hepatocytes (arrow) in CHC of low grade activity and low stage fibrosis (**A**), both nuclear and cytoplasmic expression in high grade and stage CHC (**B**), marked cytoplasmic with focal nuclear expression in low grade HCC (**C**), marked cytoplasmic and nuclear expression in high grade HCC (**D**). Histone H3-mRNA- ISH, Original magnification 40×
CHC – chronic hepatitis C, HCC – hepatocellular carcinoma

Table V. Expression of histone H3 in different grades and stages of chronic hepatitis C

Chronic hepatitis C	No of patients	Nuclear expression Mean ± SEM	Cytoplasmic expression Mean ± SEM
Low grade (GO-GI)	20	72.08 ±22.10	63.18 ±25.52
High grade (GII-GIII)	10	67.14 ±19.97	67.86 ±27.06
Low stage (SI-II)	21	75.55 ±14.32	64.28 ±26.83
High stage (SIII-IV)	9	63.25 ±19.29	68.11 ±27.54

Table VI. Expression of histone H3 in different grades of hepatocellular carcinoma

Hepatocellular carcinoma	No of patients	Nuclear expression Mean ± SEM	Cytoplasmic expression Mean ± SEM
Low grade (GI-GII)	12	53.43 ±29.44	70.05 ±20.14
High grade (GIII-GIV)	8	51.35 ±32.15	92.33 ±20.66[a]

[a]$p > 0.01$ vs. low grade

completed or inhibited, histone mRNAs are selectively and rapidly degraded with a half-life of 10 min [38]. Thus the visualization of histone mRNA expressing cells can be used to evaluate the proportion of cells in the S-phase. Histone H3 gene expression has been shown to correlate with the mitotic index of tumor cells [36, 38].

In general, the incidence of proliferating cells parallels that of carcinogenesis [40, 41]. Our finding that as the differentiation of HCC decreased, the H3-mRNA increased is in agreement with that of Nagoa *et al.* [29], who demonstrated that H3 was significantly increased in less-differentiated HCCs, implying that in HCCs, the tumor cells have accomplished cell proliferative activity during tumor progression. Our result was also in agreement with similar results that have been also reported for primary mammary [42], easophageal [43], prostate cancers [44] and Bowen's disease [20]. It is of utmost importance in our study that the H3-mRNA which might represent the tumor-cell fraction in S-phase among a proliferating-cell population in G_1, S, G_2 or M phase- was significantly higher ($p < 0.01$) in high grade HCC than in low grade of differentiation. In pancreatic ductal adenocarcinoma, the S-phase fraction as defined by the H3-mRNA labeling index increased as the degree of differentiation decreased [23]. Possible alterations in tumor-suppressor genes, which have been reported to correlate with the proliferation activity in tumor cells [29, 45], might induce more efficient DNA replication in the less-differentiated carcinomas [29].

In conclusion, H3-mRNA-ISH (which may identify S-phase cells in formalin-fixed, paraffin-embedded hepatic tissue), should promote a better understanding of the biological and clinical behavior of HCC. Thus, Histone H3 *in situ* hybridization could offer broader perspectives on the biology of cell proliferation. As histone H3 mRNA expression, was correlated with histologic grades, it could be used as an early predictor for tumorgenic changes in chronic hepatitis C, as well as an indicator of a malignant potentiality of HCCs.

References

1. Parkin D, Bray F, Ferlay J, Pisani P. Estimating the word cancer burden: Globocan 2000. Int J Cancer 2000; 94: 153-6.
2. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma carcinomas by gene expression profiling. Hematology 2004; 40: 667-76.
3. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002; 31: 339-46.
4. Yoon SK. Recent advances in tumor markers of human hepatocellular carcinoma. Intervirology 2008; 51 Suppl. 1: 34-41.
5. Hayashi J, Aoki H, Kajina K, et al. Hepatitis C virus core protein activates the MAPK/ERR cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha. Hepatology 32: 958-961.
6. Lee JS, Thorgeirsson SS. Functional and genomic implications of global gene expression profiles in cell lines from human hepatocellular cancer. Hepatology 2002; 35: 1134-43.
7. Parkin D. Global cancer statistics in the year 2000. Lancet Oncol 2002; 2: 533-43.
8. Chen N, Bai L, Li L, et al. Apoptosis pathway of liver cells in chronic hepatitis. World J Gastroenterol 2004; 10: 3201-4.
9. Van Hooser A, Goodrich DW, Allis CD, et al. Histone H3 phosphorylation is required for the initiation but not maintenance of mammalian chromosome condensation. J Cell Sci 1998; 111: 3497-506.
10. Prigent C, Dimitrov S. Phosphorylation of serine so in histone H3, what for? J Cell Sci 2003; 166: 3677-85.
11. Liu YH, Pei RJ, Cheng CC, et al. HCC CKs are altered histones during tumor transformation in hepatoma. Res Commun Mol Pathol Pharmacol 2002; 112: 27-38.

12. Togashi T, Obata M, Aoyagi Y, et al. Two distinct methods analysing chromatin structure using centrifugation and antibodies to modified histone H3: Both provide similaire chromatin states of the Rit 1/bcl 116 gene. Mol Cell Biol 2004; 22: 8215-25.

13. Chadee DN, Hendzel MJ, Tylipski CP, et al. Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. J Biol Chem 1999; 274: 24914-20.

14. Choy JS, Kron SJ. NuA4 subunit YngG2 function in intra S-phase DNA damage response. Mol Cell Biol 2002; 22: 8215-25.

15. Timmermann S, Lehrmann H, Polesskaya A, Harel-Bellan A. Histone acetylation and diseases. Cell Moll Life Sci 2001; 58: 728-36.

16. Kim DH, Kim M, Kwon HJ. Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. J Biochem Mol Biol 2003; 38: 110-9.

17. Yamashita Y, Shimada M, Harimato N, et al. Histone deacetylase inhibitor trichostatin induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 2003; 103: 572-6.

18. Emanuele S, Lauricella M, Tesoriere G. Histone deacetylase inhibitors: apoptotic effects and clinical implications. Int J Oncol 2008; 33: 637-46.

19. Sakamoto R, Nitta T, Kamikawa Y, et al. The assessment of cell proliferation during 9, 10-dimethyl-1, benzan-thracence-induced hamster-tongue carcinogenesis by means of histone H3mRNA in situ hybridization. Med Electron Microsc 2004; 37: 52-61.

20. Murakami M, Mizoguchi Y, Horibe Y, et al. In situ localization of S-phase specific histone (H3) mRNA in Bowen's disease. APMIS 1999; 107: 1005-12.

21. Spencer VA, Davie JR. Signal transduction pathways and chromatin structure in cancer cells. J Cell Biochem Suppl 2000; 35: 27-35.

22. Gown AM, Jiang JJ, Matles H, et al. Validation of S-phase specificity of histone (H3), in situ hybridization in normal and malignant cells. J Histochem Cytochem 1996; 44: 221-6.

23. Arakura N, Hayama M, Honda T, et al. Histone H3 mRNA in situ hybridization for identifying proliferating cells in human pancreas, with special references to the ductal system. Histochem J 2001; 3: 183-91.

24. World Medical Association. Declaration of Helsinki: ethical principles for medical research involving human subjects. Available at: http://www.wma.net/e/policy/pdf/17c.pdf Accessed September 4, 2008.

25. Saber M, Omar M, Badwai H, Romeih M. Comparative studies in serological diagnosis of hepatitis C virus infection. J Hepatol Gastroenterol Inf Dis 1995; 3: 47-51.

26. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology 1994; 20: 15-20.

27. Ishak KG. Virus interference: The interferon. Proc R Soc Lond B Biol Sci 1994; 147: 258-267.

28. Maeyama H, Furuwatari C, Ota H, Akamatsu T, Nakayama J, Katsuyama T. Histone H3 messenger RNA in situ hybridization for identifying proliferating cells in formalin-fixed rat gastric mucosa. Histochem J 1997; 29: 867-73.

29. Nagao T, Inshida Y, Kondo Y. Determination of S-phase cells by in situ hybridization for histore H3 mRNA in hepatocellular carcinoma: Correlation with histologic grade and other cell proliferative markers. Mod Pathol 1996; 9: 99-104.

30. Maurer HR. Potential pitfalls of (3H) thymidine technique to measure cell proliferation. Cell Tissue Kinetics 1981; 14: 111-20.

31. Venturi A, Piaz FD, Giovannini C, Gramantieri L, Chieco P, Bolondi L. Human hepatocellular carcinoma expresses specific PCNA isoforms: An in vivo and in vitro evaluation. Lab Invest 2008; 88: 995-1007.

32. Morris GF, Mathews MB. Regulation of proliferating cell nuclear antigen during the cell cycle. J Biol Chem 1989; 264: 13856-64.

33. Coltera MD, Gown AM. PCNA/Cyclin expression and Brdu uptake define different subpopulations in different cell lines. J Histochem Cytochem 1991; 39: 23-30.

34. Bravo R, Macdonald-Bravo H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: Association with DNA replication sites. J Cell Biol 1987; 105: 1549-54.

35. Chou MY, Chang AL, McBride J, Donoff B, Gallagher GT, Wong DT. A rapid method to determine proliferation patterns of normal and malignant tissues by H3 mRNA in situ hybridization. Am J Pathol 1990; 136: 729-33.

36. Rautiainen E, Haapasalo H, Sallinen P, Rantala I, Helen P, Helin H. Histone mRNA in situ hybridization in astrocytomas: A comparison with PCNA, MIB-1 and mitosis in paraffin-embedded material. Histopathology 1998; 32: 43-50.

37. Shrumperli D. Cell-cycle regulation of histone gene expression. Cell 1986; 45: 471-2.

38. Heintz N, Sive H, Roeder R. Regulation of human histone gene expression: Kinetics of accumulation and changes in the rate of synthesis and in the half- lives of individual histone mRNA during the HeLa cell cycle. Mol Cell Biol 1993; 3: 539-50.

39. Stenger JE, Mayr GA, Mann K, Tegtmeyer P. Formation of stable P53 homotetramer and multiples of tetramers. Mol Carcinog 1992; 5: 102-6.

40. Butterworth B, Goldsworthy T. The role of cell proliferation in multistage carcinogenesis. Proc Soc Exp Biol Med 1991; 198: 683-7.

41. Marks F, Fürstenberger G, Müller-Decker K. Tumor promotion as a target of cancer prevention. Rec Results Cancer Res 2007; 174: 37-47.

42. Beerman H, Smit VT, Kluin PM, et al. Flow cytometric analysis of DNA stem line heterogeneity in primary and metastasis breast cancer. Cytometry 1991; 12: 147-54.

43. Kaketani K, Saito T, Kuwahara A, et al. DNA stem line heterogeneity in esophageal cancer accurately identified by flow cytometric analysis. Cancer 1993; 72: 3564-70.

44. Kucuk O, Demirer T, Gilman-Sachs A, et al. Intratumor heterogeneity of DNA ploidy and correlations with clinical stage and histologic grade in prostate cancer. J Surg Oncol 1993; 54: 171-4.

45. Campani D, Boggi U, Cecchetti D, et al. P53 over expression in lymph node metastases predicts clinical outcome in ductal pancreatic cancer. Pancreas 1999; 19: 26-32.