

ORIGINAL ARTICLES

Proliferating cell nuclear antigen (PCNA) activity in hepatocellular carcinoma, benign peri-neoplastic and normal liver

Kein-Seong MUN *MBBS, MPath*, Phaik-Leng CHEAH *MBBS, FRCPath*, Nurul Bahiyah BAHARUDIN *MBBS, MPath* and Lai-Meng LOOI *FRCPath, FRCPA*

Department of Pathology, Faculty of Medicine, University of Malaya, Kuala Lumpur

Abstract

Hepatocellular carcinoma (HCC) is among the ten most common cancers in Malaysian males. As cellular proliferation is an important feature of malignant transformation, we studied the proliferation pattern of normal and benign perineoplastic liver versus hepatocellular carcinoma in an attempt to further understand the tumour transformation process. 39 HCC (21 with accompanying and 18 without cirrhosis) histologically diagnosed at the Department of Pathology, University of Malaya Medical Centre between January 1992 and December 2003 were immunohistochemically studied using a monoclonal antibody to PCNA (Clone PC10: Dako). 20 livers from cases who had succumbed to traumatic injuries served as normal liver controls (NL). PCNA labeling index (PCNA-LI) was determined by counting the number of immunopositive cells in 1000 contiguous HCC, benign cirrhotic perineoplastic liver (BLC), benign perineoplastic non-cirrhotic (BLNC) and NL cells and conversion to a percentage. The PCNA-LI was also expressed as Ojanguren *et al*'s grades. PCNA was expressed in 10% NL, 38.9% BLNC, 76.2% BLC and 71.8% HCC with BLNC, BLC and HCC showing significantly increased ($p < 0.05$) number of cases which expressed PCNA compared with NL. The number of BLC which expressed PCNA was also significantly increased compared with BLNC. PCNA-LI ranged from 0-2.0% (mean=0.2%) in NL, 0-2.0% (mean=0.3%) in BLNC, 0-3.6% (mean=0.7%) in BLC and 0-53.8% (mean=7.6%) in HCC with PCNA-LI significantly increased ($p < 0.05$) only in HCC compared with BLC, BLNC and NL. Accordingly, all NL, BLC and BLNC showed minimal (<5% cells being immunopositive) immunoreactivity on Ojanguren *et al*'s grading system and only HCC demonstrated immunoreactivity which ranged up to grade 3 (75% of cells). From this study, there appears to be a generally increasing trend of proliferative activity from NL to BLNC to BLC and HCC. Nonetheless, BLNC and BLC, like NL, retained low PCNA-LI and only HCC had a significantly increased PCNA-LI compared with the benign categories. This is probably related to the malignant nature of HCC and may reflect the uncontrolled proliferation of the neoplastic hepatocytes.

Key words: PCNA, liver, normal, perineoplastic, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignant primary tumour in the liver.¹ It is estimated that the annual incidence is 1.2 to 2.5 million worldwide.² There is a distinct geographical variability in its incidence with HCC being relatively uncommon in the West but prevalent in tropical Africa and South East Asia.^{3,4} In Malaysia, HCC is among the ten most common cancers in the male population.⁵ As cellular proliferation is an important feature in malignant transformation, we were interested to

assess whether proliferative activity in the liver altered significantly during transformation from normal to HCC. Admittedly, the spectrum of conditions that precede malignancy in the liver has not been clearly elucidated. Nevertheless, cirrhosis, dysplastic nodules, nodular regenerative hyperplasia etc have been alluded to as precursor lesions⁶⁻⁸ and for the purpose of this study, we worked on the premise that benign perineoplastic liver, cirrhotic or otherwise, had "experienced" similar insults as their neoplastic counterpart, and was considered intermediate states in the

transformation spectrum. On the contrary, livers harvested from individuals who had succumbed to traumatic injuries and with no known liver disease constituted “normal” controls. Proliferative activity was immunohistochemically determined using antibodies to proliferating cell nuclear antigen (PCNA), a 36-kilodalton nuclear protein synthesized in the G1/S-phase of the cell cycle which is associated with DNA synthesis and repair.⁹⁻¹² Although some work has been done in this area, most workers have either focused on studying the rate of PCNA expression in the different liver diseases^{13,14} or took to deriving the labeling index by counting the actual number of cells which expressed PCNA in the different liver diseases.^{15,16} While both of the above methods provide different angles to understanding proliferation in the liver as it progresses from non-neoplastic to neoplastic, we incorporated both methods of analysis in the attempt to obtain further insight.

MATERIALS AND METHODS

All cases of HCC histologically diagnosed at the Department of Pathology, University of Malaya Medical Centre between January 1992 and December 2003 and had undergone hepatectomy or wedge resection of the tumour were retrieved from the files. The slides of all the cases were retrieved and histologically reviewed. Only histologically re-confirmed cases were considered for the study. One paraffin block containing both HCC and benign perineoplastic liver tissue [including cirrhotic (BLC) and non-cirrhotic (BLNC)] was selected for immunostaining for each case. A wedge biopsy of macroscopically normal liver obtained

at autopsy from 20 cases who had succumbed to traumatic injuries and with no known or any previously documented liver disease were used as normal liver (NL) controls. Except for mild congestion, all the NL did not have any significant pathology on histological examination.

4 μ m sections were cut from each of the paraffin blocks of the HCC with their surrounding perineoplastic liver and NL cases onto sialinised slides for immunohistochemical staining using the monoclonal mouse anti-proliferating cell nuclear antigen (Clone PC10 : Dako) at a dilution of 1:800 with the DakoCytomation EnVision+ System-HRP (DAB) kit according to the manufacturer’s instructions. After deparaffinisation and rehydration, the tissue sections were quenched with Peroxidase Block supplied in the kit. The sections were then incubated with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins in 2 sequential 30-minute incubations and finally visualised via diaminobenzidine chromogen. Tonsillar tissue with known immunoreactivity for PCNA served as positive control. The negative control consisted of the above tonsillar tissue immunostained in the same manner but with substitution of distilled water for the primary antibody. Both positive and negative controls were run with each batch. Only unequivocal nuclear positivity was interpreted as immunopositive for PCNA (Fig 1). The PCNA labeling index (PCNA-LI) was determined by counting the number of PCNA labeled nuclei in 1000 contiguous HCC, BLC, BLNC and NL cells using an Olympus BX51 light microscope and subsequently expressed as a percentage. The PCNA-LI was also accordingly translated into

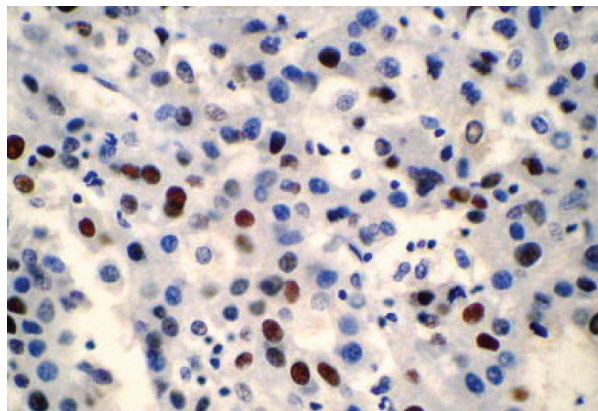


FIG. 1: Hepatocellular carcinoma with nuclear PCNA expression

Ojanguren *et al*'s grades,¹⁷ with grades varying from absent (immunonegative); minimal (<5%); grade 1 (5-25%); grade 2 (26-50%); grade 3 (51-75%) and grade 4 (>75%) immunoreactivity. Analysis of results was carried out using SPSS 11.5 statistical package.

RESULTS

Thirty-nine HCC were finally admitted into the study. The benign perineoplastic liver was cirrhotic in 21 and non-cirrhotic in 18. The ages of the patients ranged from 40 years to 70 years, with a mean of 58.3 years. There were 30 males and 9 females (M:F = 3.3:1). Ethnically, 34 (87.2%) were Chinese, 4 (10.3%) Malay and 1 (2.6%) Indian. The ages of the NL cases ranged from 17 to 56 years (mean = 27.9 years) with 15 males and 5 females (M:F = 3:1). Ethnically, there were 12 Malays (60.0%), 5 Indians (25.0%) and 3 Chinese (15.0%). PCNA was expressed in 10.0% NL, 38.9% BLNC, 76.2% BLC and 71.8% HCC. HCC, BLC and BLNC showed significantly increased ($p < 0.05$) number of cases which expressed PCNA compared with NL. In addition, a significantly increased ($p < 0.05$) number of BLC expressed PCNA compared with BLNC. The number of cases of HCC and BLC which expressed PCNA were not significantly different. The PCNA-LI and grade of immunoreactivity for HCC, BLC, BLNC and NL are illustrated in Table 1. PCNA-LI ranged from 0-53.8% (mean=7.6%) in HCC, 0-3.6% (mean=0.7%) in BLC, 0-2.0% (mean=0.3%) in BLNC and 0-2.0% (mean=0.2%) in NL. PCNA-LI was significantly increased ($p < 0.05$) in HCC compared with both BLC and BLNC and NL. There was no significant difference in PCNA-

LI between BLC, BLNC and NL ($p > 0.05$). Using Ojanguren *et al*'s grading system, all NL, BLC and BLNC only showed minimal (<5%) immunoreactivity. In contrast, 16 (41.0%) HCC showed minimal, 8 (20.5%) grade 1, 3 (7.7%) grade 2 and 1 (2.6%) grade 3 immunoreactivity. No HCC, BLC, BLNC or NL exhibited grade 4 PCNA immunopositivity.

DISCUSSION

In this study, HCC showed a predilection for Chinese males with a mean age of 58 years. These findings are similar to that reported in the Malaysian National Cancer Registry⁵ and that of a previous study by Cheah *et al*.¹⁸

In this study, HCC, BLC and BLNC showed significantly increased ($p < 0.05$) number of cases which expressed PCNA compared with NL. From these findings, it appears that more "diseased" livers, neoplastic and non-neoplastic, expressed PCNA than "normal, undiseased livers". Nevertheless, PCNA was also expressed by 2 of twenty normal livers. The reason for this is unclear but other studies have also recorded PCNA expression in normal livers.^{17,19} It is interesting that 76.2% BLC demonstrated PCNA expression and this was significantly higher ($p < 0.05$) than BLNC (38.9%). The number of cases of BLC which expressed PCNA was marginally higher than HCC (71.8%). This probably confirms the general belief that cirrhotic livers have on-going proliferation in response to hepatocyte destruction. That BLNC was significantly more proliferative than normal liver could be attributed to several factors. It is possible that some process with resultant inducement of proliferation was still on-going in the surrounding

TABLE 1: PCNA immunoreactivity grading (Ojanguren *et al*¹⁴) and labeling index (PCNA-LI) in hepatocellular carcinoma (HCC), benign perineoplastic cirrhotic liver (BLC), benign perineoplastic non-cirrhotic liver (BLNC) and normal control liver (NL)

| | | HCC (n=39) | BLC (n=21) | BLNC (n=18) | NL (n=20) |
|-------------|---------|------------|------------|-------------|-----------|
| PCNA grade | | | | | |
| | Absent | 11 | 5 | 11 | 18 |
| | Minimal | 16 | 16 | 7 | 2 |
| | 1 | 8 | 0 | 0 | 0 |
| | 2 | 3 | 0 | 0 | 0 |
| | 3 | 1 | 0 | 0 | 0 |
| | 4 | 0 | 0 | 0 | 0 |
| PCNA-LI (%) | Range | 0-53.8 | 0-3.6 | 0-2.0 | 0-2.0 |
| | Mean | 7.6 | 0.7 | 0.3 | 0.2 |

non-cirrhotic perineoplastic liver or alternatively, as suggested by some workers, that HCC itself could elaborate factors which could directly stimulate or derepress PCNA expression in normal cells within its vicinity.²⁰⁻²² In terms of PCNA-LI, only HCC showed significant increase ($p < 0.05$) compared with BLC, BLNC and NL. This was also reflected in the immunoreactivity grading whereby all the benign livers (NL, BLC and BLNC) expressed PCNA in minimal amounts (<5% of cells) compared with HCC which demonstrated PCNA expression from minimal to grade 3 (51-75% of cells).

Taken together, there is a generally increasing trend of proliferative activity from NL to BLNC to BLC and HCC. Nonetheless, non-neoplastic livers, NL, BLNC, and BLC demonstrated significantly lower PCNA-LI with means of 0.2%, 0.3% and 0.7% respectively compared with 7.6% in HCC. While the rate of BLC expressing PCNA parallels that of HCC, PCNA-LI of the former was significantly lower than the latter. The finding of lower PCNA-LI in cirrhosis compared with HCC is similar to that in other studies.^{15,16,23} It appears that the malignant nature of HCC sets it aside in terms of proliferative activity and the increased frequency and high PCNA-LI of HCC may reflect the uncontrolled proliferation of the monoclonal neoplastic hepatocytes. The high frequency of PCNA expression but low PCNA-LI in cirrhosis probably means that in most cases of cirrhosis there are small numbers of hepatocytes which are undergoing controlled regenerative proliferation.

ACKNOWLEDGEMENT

This study was funded by the Malaysian Ministry of Science, Technology and Innovation IRPA research grant : 06-02-03-0071-PR0019/04-2

REFERENCES

1. Ferrel LD, Geisenger KR. Surgical Diseases of the Liver. In: Silverberg SG, DeLellis RA, Frable WJ, LiVolsi VA, Wick MR, editors. *Silverberg's Principles and Practice of Surgical Pathology and Cytopathology*; Churchill Livingstone – Elsevier, 4th edition 2006. Volume 2, Chapter 29, p. 1527 – 1547.
2. Idilman R, De Maria N, Colantoni A, Van Thiel DH. Pathogenesis of hepatitis B and C induced hepatocellular carcinoma. *J Viral Hepatitis* 1998; 5: 285 – 99
3. Desmet VJ, Rosai J. Liver. In: Rosai J, editor. *Rosai and Ackerman's Surgical Pathology*; Mosby, 9th edition 2004. Volume 1, Chapter 13, p. 917 – 1033.
4. Anthony PP. Tumours and tumour-like lesions of the liver and biliary tract: aetiology, epidemiology and pathology. In: MacSween RNM, Burt AD, Portmann BC, Ishak KG, Scheuer PJ, Anthony PP, editors. *Pathology of the Liver*; Churchill Livingstone, 4th edition 2002. Chapter 15, p. 711 – 775.
5. Lim GCC, Halimah Y. 2nd Report of the National Cancer Registry: Cancer incidence in Malaysia 2003. National Cancer Registry, Malaysia, 2004
6. Imanishi H, Cheng J, Ikeda N, Saito M, Ohno M, Hara N *et al.* Evaluation of interleukin-12-induced interferon- γ production in vitro by peripheral blood mononuclear cells in patients with chronic liver disease. *Hepatogastroenterology* 2003; 50: 1502-5.
7. Park YN. Pathology of hepatocellular carcinoma: recent update. *Korean J Gastroenterol* 2005; 45:227-33
8. Oh BK, Kim YJ, Park YN, Choi J, Kim KS, Park C. Quantitative assessment of hTERT mRNA expression in dysplastic nodules of HBV-related hepatocarcinogenesis. *Am J Gastroenterol.* 2006; 101:831-8.
9. Kurki P, Ogata K, Tan EM. Monoclonal antibodies to proliferating cell nuclear antigen (PCNA/cyclin) as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *J Immunol Methods* 1988; 109: 49 – 59
10. Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell* 1992; 69: 367 – 74
11. Waseem NH, Lane DP. Monoclonal analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J Cell Sci* 1990; 96: 121 – 9
12. Gramantieri L, Trete D, Chieco P, Lacchini M, Giovannini C, Piscaglia F *et al.* In human hepatocellular carcinoma in cirrhosis proliferating cell nuclear antigen (PCNA) is involved in cell proliferation and cooperates with P21 in DNA repair. *J Hepatol.* 2003; 39: 997 – 1003
13. Fu XM, Yang QX, Shao CK, Feng ZY. Expression of h-TERT, c-myc, PCNA and cell apoptosis in liver carcinogenesis. *Nan Fang Yi Ke Da Xue Xue Bao* 2006; 26:821-3
14. Shen LJ, Zhang HX, Zhang ZJ, Li JY, Chen MQ, Yang WB *et al.* Detection of HBV, PCNA and GST-pi in hepatocellular carcinoma and chronic liver diseases. *World J Gastroenterol* 2003; 9: 459-62
15. Lake-Bakaar G, Mazzocchi V, Ruffini L. Digital image analysis of the distribution of proliferating cell nuclear antigen in hepatitis C virus-related chronic hepatitis, cirrhosis and hepatocellular carcinoma. *Dig Dis Sci* 2002; 47: 1644-8
16. Park YN, Chae KJ, Kim YB, Park C, Theise N. Apoptosis and proliferation in hepatocarcinogenesis related to cirrhosis. *Cancer* 2001; 92:2733-8
17. Ojanguren I, Ariza A, Llatjos M, Castella E, Mate JL, Navas-Palacios JJ. Proliferating cell nuclear antigen expression in normal, regenerative and neoplastic liver: A fine needle aspiration cytology and biopsy study. *Hum Pathol* 1993; 24: 905-8
18. Cheah PL, Looi LM, Nazarina AR, Goh KL, Rosmawati M, Vijeyasingam R. Histopathological

- landmarks of hepatocellular carcinoma in Malaysians. *Malays J Pathol* 2003; 25:37-43
19. Harrison RF, Reynolds GM, Rowlands DC. Immunohistochemical evidence for the expression of proliferating cell nuclear antigen (PCNA) by non-proliferating hepatocytes adjacent to metastatic tumours and in inflammatory conditions. *J Pathol* 1993; 171:115-22
 20. Hall PA, Coates PJ, Goodlad RA, Hart IR, Lane DP. Proliferating cell nuclear antigen expression in non-cycling cells may be induced by growth factors in vivo. *Br J Cancer* 1994; 70:244-7
 21. Nan KJ, Ruan ZP, Jing Z, Qin HX, Wang HY, Guo H *et al.* Expression of fragile histidine triad in primary hepatocellular carcinoma and its relation with cell proliferation and apoptosis. *World J Gastroenterol* 2005; 11:228-31
 22. Tsuboi Y, Ichida T, Sugitani S, Genda T, Inavoshi J, Takamura M *et al.* Overexpression of extracellular signal-regulated protein kinase and its correlation with proliferation in human hepatocellular carcinoma. *Liver Int* 2004; 245:432-6
 23. Nakajima T, Kagawa K, Ueda K, Ohkawara T, Kimura H, Kakusui M *et al.* Evaluation of hepatic proliferative activity in chronic liver diseases and hepatocellular carcinomas by proliferating cell nuclear antigen (PCNA) immunohistochemical staining in methanol-fixed tissues. *J Gastroenterol* 1994; 29:450-4