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Original Paper

Altered Expression of the Growth and Transformation Suppressor *PML* Gene in Human Hepatocellular Carcinomas and in Hepatitis Tissues

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The promyelocytic leukaemia (*PML*) gene, which encodes a transformation and growth suppressor, was first identified at a chromosomal translocation break point in acute promyelocytic leukaemia. To elucidate if *PML* may be involved in hepatocellular carcinoma (HCC), the expression of *PML* was analysed using immunohistochemistry in human HCC and hepatitis tissues. Our studies demonstrated overexpression of PML protein in the PML-oncogenic domain (POD) structure in 50% of HCC (11/22). Enhanced expression and cytoplasmic localisation of PML was associated with cirrhosis. Increased expression of PML was also found in liver abscesses. However, in colon metastasis to the liver, the expression of PML was moderate to low, although strong expression was seen in the surrounding interstitial cells, macrophages and lymphocytes, an indication of the inflammation process associated with tumour growth. Most interestingly, strong expression of PML was found in neoplastic cells at the periphery of the tumours, but progressively decreased in cells at the centre of the tumours, which may be associated with an altered transform phenotype or apoptosis. The altered expression of PML indicates that this nuclear protein may play an important role in cellular response to stress and inflammation, as well as in compensatory cell growth. © **1998 Elsevier Science Ltd. All rights reserved**

Key words: suppressor genes, PML, hepatocellular carcinoma, hepatitis B, gene expression regulation, neoplastic

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INTRODUCTION

Disruptions of proto-oncogenes and oncosuppressor genes have been attributed to the pathogenesis of many neoplastic diseases [1–4]. The promyelocytic leukaemia (*PML*) gene encodes a growth and transformation suppressor, and has been identified at the non-random chromosomal translocation break point t(15;17)(q22;q12) of acute promyelocytic leukaemia (APL) [5–8]. This translocation event fuses the *PML* and the retinoic acid receptor-α (*RARA*) genes and encodes the PML–RARA and RARA–PML fusion proteins. *PML–RARA* apparently exerts a dominant negative effect in cells by inactivating the normal functions of PML and retinoic acid X receptor (RXR) proteins.

PML is a nuclear phosphoprotein which has been shown to inhibit cell growth and the transformed phenotypes of tumour cells [9–12]. PML has also been found to accumulate at the border of a nuclear structure, namely the nuclear bodies or PML oncogenic domain (POD) [13–16]. In APL cells, the PML–RARA fusion protein was found to accumulate into aberrant microstructures and is able to delocalise some other nuclear proteins. Thus, PML is a transcriptional factor that has been considered to play a crucial role in the pathogenesis of APL. Moreover, there is evidence that PML is involved in cell cycle progression and viral replication [9, 13–18]. A significant increase in PML nuclear bodies and a non-aggregated soluble form was found as cells progress through G1

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and S phases of the cell cycle [18]. PML is also a member of a novel family of ring-finger proteins and is structurally related to enzymes expressing DNA repair and DNA recombination activities [15–17].

Although reports on PML in other cancers have been documented [17–20], the precise role of PML in regulating cell growth and transformed phenotypes of other neoplasias, including the hepatocellular carcinomas (HCCs), is still unclear. HCC accounts for approximately 85% of primary malignant tumours of the liver and HCC also shows marked geographical variation in incidence [21]. In Hong Kong and South-eastern Asia, with an incidence of 25–35/100 000, it is one of the major cancers in this region, especially in those with hepatitis B virus infection and/or liver cirrhosis. Our laboratories are interested in the aetiology, diagnosis and treatment of neoplastic diseases including HCC [20–23], and to elucidate if *PML* resembles other oncosuppressor genes such as *p53* and *Rb*, we examined the expression of PML protein in human tissues of HCC, hepatitis, cirrhosis, liver abscess and secondary liver cancers.

PATIENTS AND METHODS

Patients and samples

All samples were from Hong Kong Chinese patients. Twenty-two samples of HCC were obtained which included five well differentiated HCC, six well to moderately differentiated or moderately differentiated HCC, four moderately to poorly or poorly differentiated HCC and seven unspecified or recurrent HCC. Of these, 14 were related to chronic hepatitis B virus infection. As control tissues, six samples of secondary liver tumours (metastatic adenocarcinoma from colon), six samples of chronic hepatitis infected liver with cirrhosis and two samples from liver abscesses were used. Eighteen normal samples of liver tissue (from autopsy or transplanted liver) were also studied.

Immunohistochemical staining

Immunohistochemical staining was analysed using affinity-purified rabbit antibody raised against the recombinant PML protein, as described previously [9–12]. In brief, tissue samples were obtained from resected livers and tumours, and were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. Tissue sections were prepared in polylysine coated slides and all steps were performed at room temperature. Slides were first heated at 60°C in an oven. After dewaxing with xylene and rehydration with gradient alcohols, the slides were heated to boiling in 10 mM sodium citrate buffer pH 6.8 for 10 min in a microwave oven. After treatment with 3% hydrogen peroxide in methanol to inactivate the endogenous peroxidase activity, the samples were blocked with the blocking solution which consisted of 5% bovine serum albumin (BSA) in PBS, for 20 min, and then incubated with the first antibody, PML antibody (1:2000 in blocking solution), for 1–2 h at room temperature. After washing three to four times with PBS, the samples were incubated with each secondary antibody (goat anti-rabbit IgG-conjugated with biotin and then with avidin-conjugated with horse radish peroxidase, BioGenex Lab., California, U.S.A.) for 30 min, and washed with PBS after each step. The slides were developed with substrates for peroxidase (AEC or DAB reagents, Histostain kit, Zymed Lab, S. San Francisco, California, U.S.A.). After counterstaining with haematoxylin and briefly washing with 30 mM ammonium hydroxide, the slides were mounted with aqueous or permanent mounting medium. The expression of PML in the POD structure was rated as: +++, very strong or >20 POD; ++, strong or 10–20 POD; +, moderate or 5–10 POD; +/−, weak or partial, or 1–5 POD; and −, negative.

RESULTS

Altered expression of PML in normal liver, HCC and secondary liver tumours

In normal liver (Figure 1a) low but detectable levels of PML (14 cases +/−; 4 cases negative) were found in the nuclei of hepatocytes as POD or nuclear bodies, confirming results previously published [19]. Other cells that are known to express low levels of PML in liver include Kuppfer cells, activated macrophages, epithelia and endothelial cells [18–20]. In HCC samples a 5–10-fold increase in expression of PML was found in the tumour cells. In addition, endothelial cells, epithelia, activated macrophages, lymphoid cells and Kuppfer cells were also highly stained positive for PML. As shown in Figure 1(b), considerable enhanced expression of PML in the nucleus was found in a large number of HCC samples. The typical trabecular arrangement with endothelial-lined sinusoids separating the aggregates of tumour cells was also observed. Interestingly, in some samples of HCC, a cytoplasmic localisation of PML was observed. To determine if the enhanced expression was specific to primary liver neoplasia, several samples of secondary liver cancers were investigated. As shown in Figure 1(c), the expression of PML in secondary liver cancers, which were colon adenocarcinomas that metastasised to the liver, usually ranged from moderate to low but still detectable. However, the staining was mostly confined to the nucleus as nuclear bodies or PODs. In contrast, liver cells surrounding the cancer contained increased levels of PML, both in the nucleus and in the cytoplasm. In addition, the expression of PML was also found to increase in the infiltrating lymphocytes, macrophages and fibroblast cells.

Increased expression of PML in chronic hepatitis tissues

Although the precise relationship between hepatitis and liver cirrhosis and HCC is still uncertain, it is believed that chronic hepatitis and cirrhosis are premalignant lesions. To determine if the increased expression of PML is also associated with precancerous conditions of HCC, cirrhotic liver samples of patients with chronic hepatitis were immunostained with PML. As shown in Figure 2(a), strong nuclear staining of PML was found in the hepatocytes of the hepatitis tissues, similar to the non-tumour area of HCC slides which were usually cirrhotic in nature. However, cytoplasmic localisation of PML was also observed in hepatitis tissues. In addition, infiltrating lymphocytes and macrophages surrounding cirrhotic liver also showed strong expression of PML, which indicates that the enhanced expression of PML occurs in a variety of cell types. The staining was mostly homogeneous, uniformly distributed and with a diffuse pattern. The surrounding Kupffer cells, which are one of the sinusoidal endothelial cells and the resident macrophages in the liver, were also stained strongly by the antibody against PML. Kupffer cells have important roles in the biological defence mechanism and in the maintenance of homeostasis of the body. As shown in Figure 2(b), strong PML staining was observed in lymphocytes surrounding cirrhotic liver nodules.

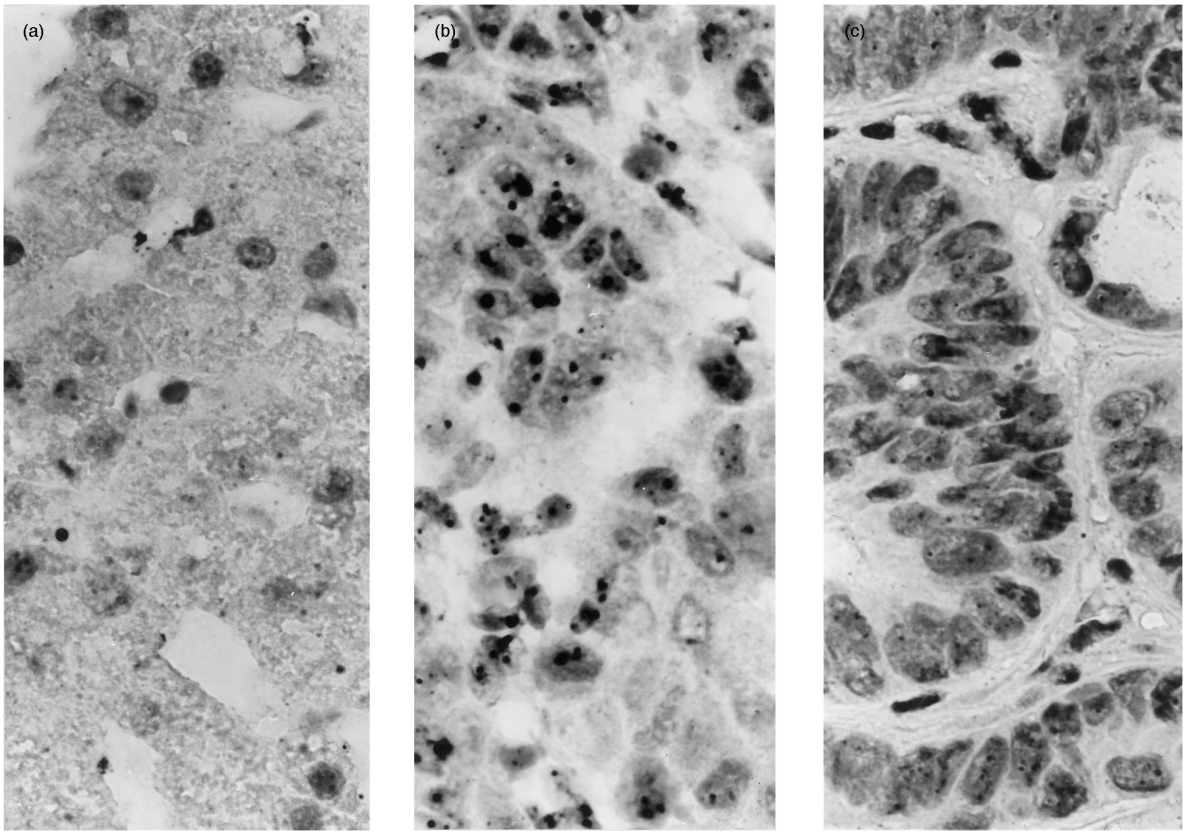

Figure 1. Expression of PML in (a) normal liver, (b) hepatocellular carcinoma (HCC) and (c) secondary liver cancer of metastasis from a colon adenocarcinoma (400×).

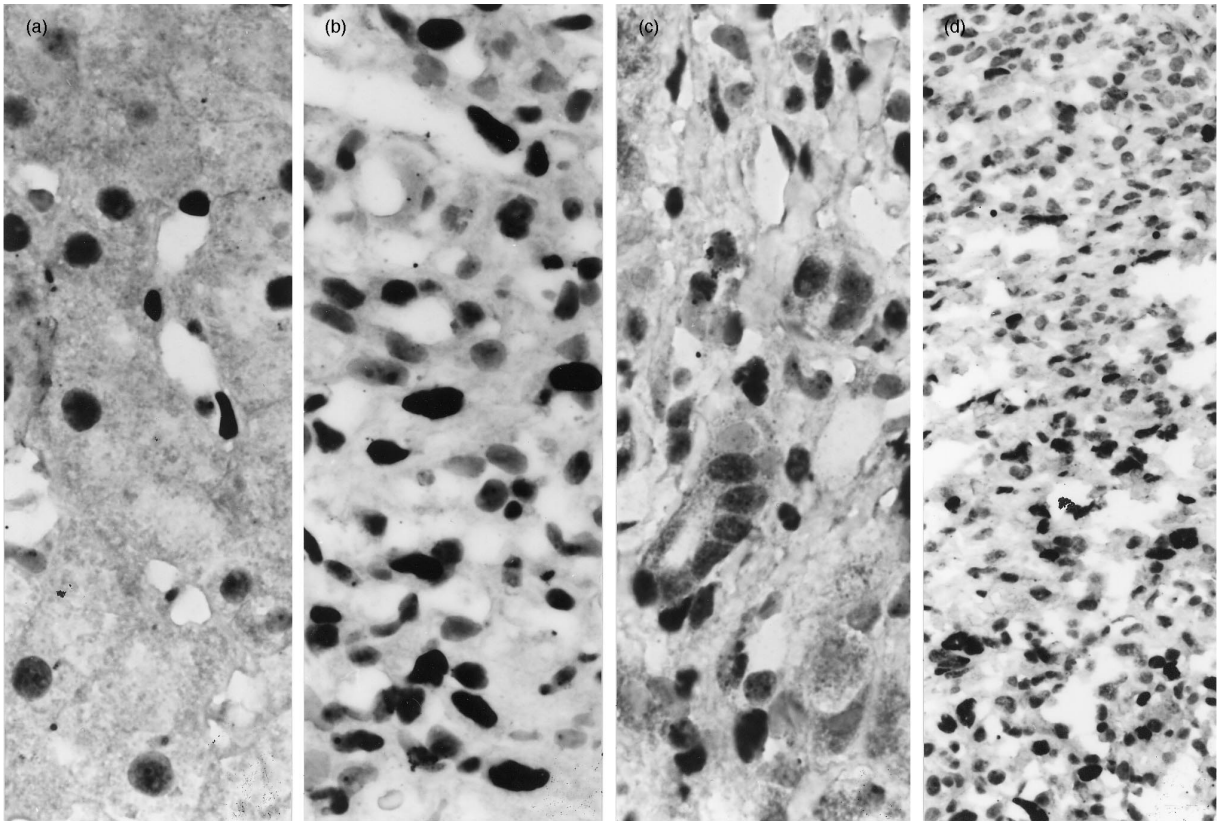

Figure 2. PML expression in different cell types of cirrhotic liver from a chronic hepatitis patient and in liver abscess. (a) Hepatocytes, (b) lymphocytes of hepatitis tissue, (c) bile duct epithelial cells of hepatitis tissues, (d) liver abscess. (400×).

Table 1. *PML expression in hepatocellular carcinoma (HCC) and secondary liver cancers*

		PML staining		
	Sample no.	Tumour centre	Peri-tumour	Non-tumour
Hepatocellular carcinoma				
Well differentiated	5	+ ($n=3$)	++ ($n=3$)	+++ ($n=2$)
		+/− ($n=1$)	+ ($n=2$)	++ ($n=3$)
		− ($n=1$)		
Well to moderately or moderately	6	++ ($n=2$)	++ ($n=2$)	+++ ($n=1$)
		+ ($n=1$)	+ ($n=3$)	++ ($n=2$)
		+/− ($n=3$)	+/− ($n=1$)	+ ($n=3$)
Moderately to poor or poorly	4	+++ ($n=2$)	++ ($n=2$)	++ ($n=2$)
		++ ($n=1$)	+ ($n=2$)	+ ($n=2$)
		+/− ($n=1$)		
Unspecified	7	+++ ($n=1$)	++ ($n=1$)	+++ ($n=1$)
		+ ($n=1$)	+ ($n=5$)	++ ($n=2$)
		+/− ($n=5$)	+/− ($n=1$)	+ ($n=4$)
Secondary liver colon metastasis	6	++ ($n=1$)	++ ($n=1$)	+++ ($n=1$)
		+ ($n=4$)	+ ($n=4$)	++ ($n=4$)
		− ($n=1$)	− ($n=1$)	+ ($n=1$)
Liver cirrhosis	6	—	—	+++ ($n=2$)
				++ ($n=4$)
Normal liver	18	—	—	+/− ($n=14$)
				− ($n=4$)

+++, very strong, ++, strong, +, moderate; +/−, weak or partial; −, negative.

In addition, the expression of PML in bile duct epithelial cells of hepatitis tissues was found to increase significantly compared with control liver. As shown in Figure 2(c), epithelial cells associated with the bile duct were strongly stained for PML, indicating that its enhanced expression is present in several cell types. To determine if the enhanced PML expression is also associated with other pathological conditions of the liver, samples of liver abscess were analysed. Interestingly, increased expression of PML was also observed in certain cells. As shown in Figure 2(d), the layer of cells surrounding the abscess contained increased levels of PML as compared with neighbouring cells. These results strongly indicate that enhanced PML expression is associated with the process of inflammation and/or cell death, most likely with the compensatory cell growth during cell injury.

The number of HCCs and secondaries analysed and their PML expression are shown in Table 1. Five cases of well differentiated HCC contained mostly moderate (+) to weak (+/−) PML expression at the tumour centre, while strong (++) to moderate (+) PML was found in peri-tumoral regions. The surrounding non-tumorous tissues, which were mostly cirrhotic, expressed high PML (+++ to ++ Table 1). Similar results were found with the well to moderately or moderately differentiated HCC. For the moderate to poor or poorly differentiated HCC and the unspecified differentiation HCC, slightly more intense staining of PML was observed. Most tumour cells in peri-tumoral regions showed strong (++) or moderate (+) PML expression. The majority of secondary liver cancers from colon metastasis only showed moderate expression (four of six were +) in both tumour centre and at peri-tumoral regions. The surrounding non-tumoral regions of most liver cancers showed very strong (+++ ($n=2$) or ++ ($n=6$)) expression of PML. For cirrhosis, two samples were +++ while four samples showed ++ expression. In contrast, the 18 normal livers all showed weak/partial or negative expression (14 +/− and four −). These data indicate that increased PML expression is associated with liver cirrhosis and HCC. Since the non-tumorigenic regions of the HCC samples were mostly cirrhotic in nature, it is not surprising that the expression of PML was also strong. However, the expression of PML appears to have no direct correlation with differentiation, although an inverse correlation was found in unspecified differentiation HCC at the tumour centre (only two of seven samples showed positivity).

Differential expression of PML at the periphery and at the centre of single encapsulated HCC

To determine whether there is a difference in the expression of PML in the peri-tumoral regions and at the centre of the tumour, HCCs with large and single encapsulated lesions were further analysed for the expression of PML. In Figure 3(a), a region between the periphery and the tumour centre of a HCC is shown. The PML staining pattern appears to have a gradation of intensity ranging from strong in the peri-tumoral region to weaker expression towards the centre of the tumour. Enhanced expression of PML was found in the neoplastic cells at the periphery of the HCC (Figure 3b), which is apparently the site of rapid proliferation and expansion. The surrounding encapsulated fibrous layers of connective tissue stroma contained numerous fibroblasts and lymphocytes that overexpressed PML (data not shown). However, the expression of PML progressively decreased to moderate or very low levels at the centre of the tumour (Figure 3c). In contrast, PML expression in HCCs with multi-encapsulated lesions or with numerous fibrosis encapsulation was usually high and did not show a gradient of diminishing expression (data not shown). Interestingly, a differential expression of PML was found in single versus multi-encapsulated regions of HCC (Table 2). A large number of single encapsulated lesions of HCC (8 of 14, 57%) showed low or no expression of PML in the tumour centre, while the majority of multiple lesions (78%) showed positive or enhanced PML expression. These results, taken together, indicate that PML expression in the liver and in HCC is

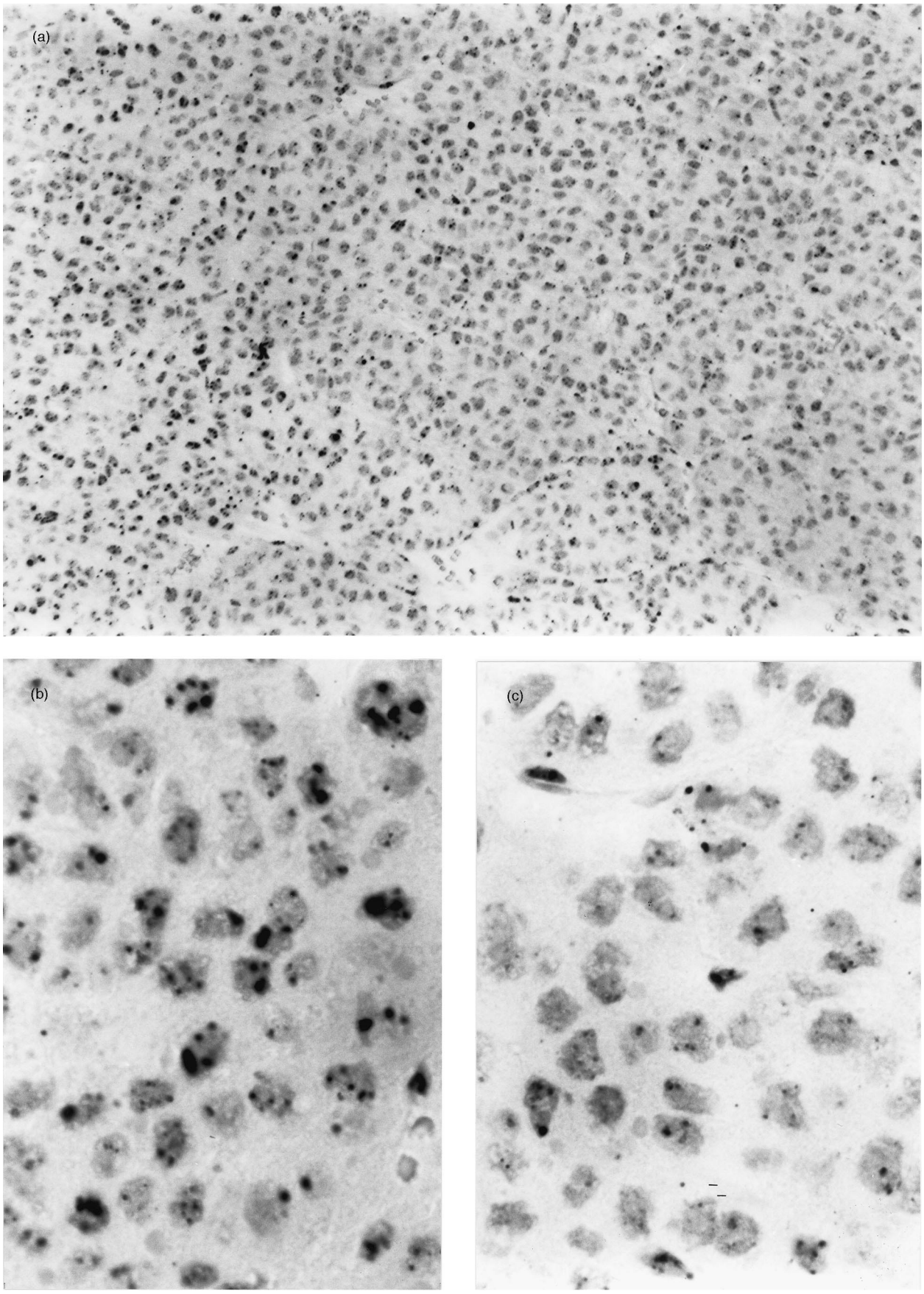

Figure 3. Gradation of expression of PML in a single encapsulated lesion of hepatocellular carcinoma (HCC). (a) ×100, (b) tumour cells at the periphery of the tumour with encapsulated fibrous tissues, (c) tumour cells located at a distance of approximately 1 cm from the periphery of the tumour (400×).

Table 2. PML expression in single and multiple encapsulated lesions of hepatocellular carcinoma

	PML staining			
	Tumour centre		Peri-tumoral	
Tumour types	Negative	Positive	Negative	Positive
Single ($n = 14$)	8 (57%)	6 (43%)	2 (14%)	12 (86%)
Multiple ($n = 9$)	2 (22%)	7 (78%)	1 (11%)	8 (89%)

rather complex, and it is apparently associated with mediators in the micro-environment of the peri-tumoral region.

DISCUSSION

Cancer development, including that of HCC, appears to be a multistage multi-hit process in which normal cells progress to malignant neoplasia through an accumulation of genetic and epigenetic changes [1–7]. Many of these genomic and phenotypic alterations occur in proto-oncogenes and onco-suppressor genes which are known to affect cellular proliferation and signal transductions [1–4]. In addition, there is considerable evidence supporting the importance of onco-suppressor genes in regulating cell growth and differentiation. Some of the tumour suppressor genes are also responsible for protecting cells from the deleterious effects of DNA damage and xenobiotics by arresting cell cycle progression and by triggering programmed cell death [3, 4]. The *p53* gene is a prototype example in which mutated forms of the gene have been found in many tumour types [4]. Overexpression of *p53* as a result of increased stability of the protein has been found in cancer cells that contained the mutated gene, or had p53 protein sequestered and inactivated by viral proteins. Mutations in specific regions of the gene may contribute to the aberrant localisation of the gene product [3]. Since not all tumours contain defective *p53*, it is believed that other molecules that regulate cell growth and differentiation may be involved in the loss of growth control during oncogenesis. PML has been found to accumulate at the border of a nuclear structure, namely the nuclear bodies or POD [10, 11, 14–16]. PML has been found to be co-localised with several proteins, including Sp-100, NDP55, PIC 1, Int-6 and possibly others, in the POD structure [24–27]. PML may play a role in viral replication [13, 14]. During adenovirus infection, the immediate early viral proteins E1B 55 kDa and E4-ORF3 11 kDa are targeted to the POD structure [13]. The Epstein–Barr virus (EBV)-encoded protein EBNA-5 has been shown to co-localise with PML in the POD structure in lymphoblastoid cells [15], while the immediate–early protein (Vmwl 10) of the herpes simplex virus (HSV) could interact with PML [16]. Whether the HBV contains a gene, the product of which may interact with PML, is at present unknown. Moreover, the specific target genes for PML are also not known, but the gene products that co-localise with PML in the POD structure may represent some of the target genes of PML, or these proteins may regulate PML. However, the precise function of PML and the POD structure is also unclear. One hypothesis put forth by Maul and co-workers [28] is that the nuclear bodies or POD may represent storage sites of certain matrix proteins readily accessible throughout the chromatin in response to stress or other effectors that induce global nuclear changes. Thus, PML may act as a matchmaker to recruit or sequester other proteins in or out of the POD. Nevertheless, PML may play an important role in the loss of growth control and in the oncogenesis of many cancers.

The gradient of expression of PML found in HCC is somewhat unexpected, but not surprising. Many regulatory molecules show a gradient of expression in tissues, cells and embryos. PML expression in HCC is probably analogous to the oxygen gradient in tumours in which the tumour centres are mostly hypoxic while the peri-tumoral regions are oxygen-rich because of the availability of ample blood supplies. Although the exact reason for the gradient of PML expression is not known, it could be an indication that cells at the peri-tumoral regions and those at tumour centres belong to different stages of the tumorigenic process. In addition, cytokines and lymphokines are known to be produced by cells in the inflammatory regions, and since PML expression can be induced by cytokines, i.e. interferons, PML may be activated in both the tumour and non-tumour cells at the peri-tumoral regions. Thus, an alternative explanation is that the gradient of expression of PML in tumours is a reflection of the differential concentration of secreted cytokine(s) at different regions of the tumours.

Our data on the enhanced nuclear and cytoplasmic expression of PML in HCC and hepatitis with an antibody raised against the recombinant protein are in general agreement with those published previously [18–20], indicating that PML displays an altered expression pattern during human oncogenesis. The studies previously reported have used a polyclonal antibody raised against the recombinant PML protein, and the 5E10 monoclonal antibody which was previously shown to recognise nuclear matrix-associated nuclear bodies. We also confirmed the results of Terris and co-workers [19] who showed that PML expression is enhanced in several cell types of the inflammatory tissues in liver because chronic inflammation and cell injury induce signals for apoptosis and regeneration, affecting cell cycle progression and the expression of PML. Inflammatory cells may increase the secretion of growth factors, cytokines and hormones. These biological mediators may in turn induce the expression of cellular stress responsive genes and growth regulatory genes, including PML, in neighbouring hepatocytes. In addition, we observed that 50% of HCC (11/22) overexpressed PML, which is similar to the results of Terris and co-workers [19] of 78% (18/23) PML positivity. However, heterogeneous nuclear expression of PML protein has also been reported in normal and neoplastic tissues [20]. Carcinomas of the larynx and skin were reported to express high levels of PML, while the expression of PML in oat cell carcinomas of the lung were reported to be low. The mechanism for the differential expression of PML in various types of tumours is at present unknown. Although some of these can be attributed to tissue-specific expression, our data, showing the differential expression of PML at various sites of a single encapsulated lesion of HCC, perhaps could resolve some of the apparent discrepancies and raise a cautionary note for the analysis of partial specimens. In contrast, multi-encapsulated lesions of HCC or tumours with numerous encapsulation and peri-tumoral fibrosis usually expressed high levels of PML, and did not show a gradient of decreasing expression. Apparently cells at the periphery are rapidly dividing and express high levels of PML, while cells at the tumour centre of the tumours, which has limited blood supply, are usually

proliferating at a slower rate and are starting to go through the process of programmed cell death. Another possibility is that cells at the centre may have a different transformed phenotype as compared with those at the peri-tumoral regions. It has been reported previously that epithelial tumours show a gradual increase in PML levels as the lesion progresses from dysplasia to carcinoma, but when malignant cells turn invasive, they lose PML expression [18].

There are apparently at least two mechanisms for the induction of PML expression, namely transcriptional regulation and post-transcriptional modification. The enhanced mRNA levels of PML after interferon induction, as reported previously [17], belongs to the former and the accumulation of PML transcripts is apparently a primary response to interferon, since it has been reported that *de novo* protein synthesis is not required. In contrast, enhanced *PML* expression after treating cells with ionising radiation is a post-transcriptional modification event, since ionising radiation arrests cells in G1, and does not involve the alteration of the mRNA level of *PML* [29]. The mechanism for the overexpression of *PML* in HCC and chronic hepatitis may involve either one or both of these two possibilities. Thus, the altered expression and localisation of PML could be the consequence of the PML protein being modified or sequestered by viral or cellular proteins including post-transcriptional modifications such as phosphorylation or dephosphorylation. Moreover, the enhanced expression of PML in these cells could be an intrinsic property of the cell types, or it could be an inducible expression as a consequence of the increased secretion of biological response modifiers. PML is known to be inducible by cytokines such as interferons and oestrogen [16–18], which are produced in response to stress and inflammation. Increased expression of PML has been found in monocyte activation or differentiation [16]. Our data on the differential expression of PML in the periphery and at the centre of HCC support the notion that enhanced PML is a reflection of inducible levels. Judging from the fact that expression of PML was strongest in the inflammatory regions of the liver, which were known to be associated with infiltration by macrophages and lymphoid cells, it is highly likely that PML levels in hepatocytes were influenced by cytokines released from the lymphoid cells. Since there is an association between liver cirrhosis in 70–90% of oriental patients with HCC, liver cirrhosis and chronic inflammation are believed to play an important role in promoting the development of HCC. Chronic inflammation and cell injury may increase the secretion of growth factors, cytokines and hormones, which in turn induce the expression of cellular genes in hepatocytes. In this context, PML is apparently playing a key role in transducing the proliferative stimuli for liver regeneration, which could also act as promoters for carcinogenesis in pre-initiated hepatocytes.

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