Overexpression of Promyelocytic Leukemia Protein and Alteration of PML Nuclear Bodies in Early Stage of Hepatocarcinogenesis

Promyelocytic leukemia protein (PML) is a major component of PML nuclear bodies (PML NBs). Fusion of promyelocytic leukemia gene (*PML*) with retinoic acid receptor α gene with the t (15;17) translocation causes disassembly of PML NBs, leading to development of acute promyelocytic leukemia. In contrast, PML overexpression as well as different morphological changes of PML NBs were described in a few solid tumors. In this study, the expression of PML through the multistep hepatocarcinogenesis was analyzed in 95 cases of human hepatocellular carcinomas (HCCs) for comparison along with dysplastic nodules (DNs) and background liver cirrhosis (LC) or chronic hepatitis by immunohistochemistry and immunoblot. In addition, cases of HCCs were further evaluated according to their histologic grade and etiology. The amount of PML as well as the number and size of PML NBs increased gradually through the progression from LC, DNs to HCCs. The overexpression of PML in HCCs was much more closely associated with HBV infection than HCV infection or alcoholic liver disease. The PML expression, however, was not correlated with histologic grade of HCCs. These results suggest that PML is involved in the early stage of multistep hepatocarcinogenesis, and HBV infection may be associated with the overexpression of PML and the morphological alteration of PML NBs.

Key Words : *Leukemia, Promyelocytic; Nuclear Body; Carcinoma, Hepatocellular; Dysplastic Nodule; Liver Cirrhosis; Hepatitis B Virus*

Ghil-Suk Yoon, Eunsil Yu

Department of Pathology, University of Ulsan College of Medicine Asan Medical Center, Seoul, Korea

Address for correspondence
Eunsil Yu, M.D.
Department of Pathology, University of Ulsan College of Medicine Asan Medical Center, 388-1 Poongnap-dong, Songpa-gu, Seoul 138-736, Korea
Tel : +82-2-2224-4552, Fax : +82-2-472-7898
E-mail : esyu@www.amc.seoul.kr

INTRODUCTION

Promyelocytic leukemia protein (PML) was first identified as the product of the promyelocytic leukemia gene (*PML*) that fuses with the retinoic acid receptor α (RARα) gene in the t (15;17) translocation of acute promyelocytic leukemia (APL) (1). PML-RARα is known to be a dominant negative oncoprotein that exerts its putative leukemogenic effect by inhibiting assembly of the distinctive nuclear structure, PML nuclear body (NB) (2). Meanwhile, immunolocalization studies using sera from patients with autoimmune diseases showed that the PML was diffusely present within the nucleus and/or localized to distinctive "multiple nuclear dots", which were originally described as an autoantigenic target in a patient with primary biliary cirrhosis (3). PML NBs vary in number between 10 and 30 per nucleus, and typically have a diameter of between 0.2 and 1 μm. The number, size, and morphology of PML NBs alter throughout the cell cycle (4, 5) and are also dynamically changed by diverse extracellular environment including DNA and RNA viral infection (6-8).

Although it is well documented the proper organization of PML NBs is essential for normal cell proliferation and hematopoietic differentiation (9), the functions of PML or PML NBs still remain elusive in the pathogenesis of inflammatory and neoplastic diseases except APL. The PML expression was considerably upregulated in inflammatory tissues such as hepatitis as well as during either normal or pathological proliferative states including benign or malignant tumors (4, 10-13). In most of malignant solid tumors examined, the PML was markedly overexpressed and organization of PML NBs was not disturbed, unlike in cases of APL (13-15). Variable PML expression in human solid tumors suggests that the PML may play another role in addition to as a tumor suppressor, inducer of apoptosis and a depot of viral proteins (16, 17), and the mechanism of PML expression can be different from that of leukemogenesis.

Among solid neoplasms in which the PML is overexpressed, we selected hepatocellular carcinomas (HCCs) by the following indications: first, the expression of PML is quite different between non-neoplastic hepatocytes and HCC cells (13, 14); secondly, most of hepatocytes are in G_0 phase of cell cycle, while a significant fraction of HCC cells are in actively proliferating stage; thirdly, hepatocellular car-

cinogenesis is typical of a multistage process from chronic liver disease including liver cirrhosis (LC) through dysplastic nodule (DN) to HCCs (18), thus the PML expression can be properly correlated with the corresponding pathologic conditions.

MATERIALS AND METHODS

Tissue samples

Ninety-five cases of surgically resected HCCs were selected from the surgical pathology files of the Department of Pathology, Asan Medical Center, Seoul, Korea or Department of Pathology, Mt. Sinai Hospital, New York University, NY, U.S.A. Seventy-five cases were positive for HBV DNA or HBs antigen. Ten cases of HCCs were positive for HCV RNA and had no evidence of HBV infection and another 10 cases had past medical history of chronic alcoholism and were negative for HCV RNA, HBs or HBc antigens. Six cases of HCCs developed within a DN and three cases had a separate DN each. Serial sections of formalin-fixed and paraffin-embedded tissues from HCCs and non-neoplastic livers were cut for hematoxylin and eosin stain and immunohistochemistry.

Histologic features of HCCs were classified according to the grading system of Edmondson and Steiner (19) and those of non-neoplastic livers were reviewed.

Among 75 cases, fresh frozen tissues from HCCs and corresponding nontumor livers were available in 9 cases, one of which had both DN and HCC.

Cell culture

HepG2, HepG2.2.15 (20) and Hep3B cells were maintained in DMEM (Gibco, Gaithersburg, MD), supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2.

Immunohistochemical and immunofluorescent staining

Immunohistochemistry was done following streptavidin biotin complex method after antigen retrieval by boiling twice in citrate buffer (pH 6.0) for 5 min as previously described (21). The primary antibody for PML used in this study was PG-M3 (Santa Cruz, California, U.S.A., 1:100 dilution). For immunofluorescent staining, cultured cells on coverslips were fixed in cold methanol for 10 min. Fluorescein-conjugated anti-mouse immunoglobulin was complexed with the primary antibodies for 1 hr. The tissue sections or cells were mounted with Fluoromount G (Fischer Scientific Co., Pittsburgh, PA) and examined by using Olympus light microscope, Olympus Venox fluorescent microscope or Zeiss confocal laser scanning microscope.

Expression of the PML was described as follows: 1) average number of PML NB-positive cells per 1,000 hepatocytes or HCC cells: 2) average number of PML NBs in one nucleus of hepatocytes or HCC cells: 3) shape of PML NBs as fine dots, large dots greater than 1.0 μm in diameter, or ball-shaped NBs.

Immunoprecipitation and immunoblotting

Preparation of nuclear proteins was done as previously described (21). Protein amount was calibrated by using BCA protein assay. Twenty μg from the supernatant of each sample was incubated with the anti-PML antibody, PG-M3 at 1:200 dilution coupled to protein A-sepharose for 1 hr at 4°C. After extensive washing with the buffer, bound proteins were separated by SDS-PAGE and transferred to nitrocellulose papers which were allowed to react with the anti-PML antibodies for 1 hr. Immune complexes were detected by the chemiluminescence detection system (Amersham Pharmacia Biotech LtD, UK).

Statistical analysis

The significance of differences in the expression of PML of HCCs, DNs, LC and chronic hepatitis (CH) was estimated by paired t-test, Two-sample test, one way analysis of variance (ANOVA) procedure. The level of statistical significance was set at 95% for all evaluations.

RESULTS

PML expression in non-neoplastic liver

Seventy-one cases of HCCs were associated with LC and 24 cases occurred in the background of chronic hepatitis (CH). In non-neoplastic liver, rare hepatocytes showed one or two PML NBs that were slightly variable in size and shape (Fig. 1A). The difference of average number of PML NB-positive hepatocytes between CH and LC was not statistically significant (Table 1). Most of the reactive hepatocytes in CH and regenerating hepatocytes in LC that revealed PML NBs were unevenly distributed and closely associated with portal inflammatory cells. Average number of PML NBs in one hepatocyte nucleus was also higher in LC than that in CH, which was not statistically significant (Table 1).

PML expression in dysplastic nodules

Nine cases of DN were associated with HCCs in the background of LC. Average number of PML NB-positive hepatocytes was significantly increased in DNs compared with that in adjacent LC or CH ($p<0.05$) (Table 1). Six of nine cases of DN were classified low grade and three were high

Table 1. Expression of the PML in hepatocellular carcinomas, dysplastic nodules, liver cirrhosis and chronic hepatitis

Diagnosis (No.of cases)	PML expression	
	No. of PML NB + cells/1,000 cells	No. of PML NB/nucleus
HCC (95)	92.81 ± 78.31[†]	3.8 ± 1.86
DN (9)	45.09 ± 46.43[‡]	2.8 ± 1.26
LC (71)	8.95 ± 7.66	1.8 ± 1.54
CH (24)	4.70 ± 6.15	0.5 ± 0.50

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body; [†]HCC vs DN, HCC vs LC and HCC vs CH, $p < 0.05$ according to paired T-test; [‡]DN vs LC and DN vs CH, $p<0.05$ according to Analysis of Variance(ANOVA) procedure: Duncan's multiple range test

Table 2. Expression of PML in 95 cases of hepatocellular carcinoma according to etiologies

Etiology (No.of cases)	PML expression	
	No. of PML NB + cells/1,000 cells	No. of PML NB/nucleus
HBV (75)	100.31 ± 83.71[†]	4.2 ± 1.84
HCV (10)	80.62 ± 39.43	2.4 ± 0.56
Alcohol (10)	48.43 ± 45.28	2.7 ± 1.79

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body; [†]HBV vs HCV and HBV vs alcoholic, $p<0.05$ according to one way Analysis of Variance (ANOVA) procedure

Table 3. Expression of the PML in 95 cases of hepatocellular carcinomas according to histologic grade

Histologic grade (No.of cases)	PML expression	
	No. of PML NB + cells/1,000 cells	No. of PML NB/nucleus
1 (12)	93.75 ± 106.5	4.5 ± 2.40
2 (40)	99.37 ± 80.93	3.5 ± 1.72
3 (33)	88.43 ± 68.25	3.8 ± 1.80
4 (10)	80.62 ± 65.31	4.6 ± 1.64
Total cases (95)	92.81 ± 78.31	3.8 ± 1.86

*HCC, hepatocellular carcinoma; DN, dysplastic nodule; LC, liver cirrhosis; CH, chronic hepatitis; PML, promyelocytic leukemia protein; PML NB, PML nuclear body

grade. There was also a difference in number of PML NB-positive hepatocytes between low (31.27 ± 43.26/1,000 cells) and high grade DNs (72.72 ± 29.69/1,000 cells) ($p<0.05$ by paired t-test). The number of PML NBs in one hepatocyte nucleus was higher in DN than in LC, which was not statistically significant. Most of PML NBs were enlarged and some of them were ball-shaped with an empty center (Fig. 1B).

PML expression in hepatocellular carcinomas

The average number of PML NB-positive HCC cells per 1,000 HCC cells was markedly increased. The differences in average number of PML NB-positive cells between HCC, DN and LC were statistically significant (Table 1). The number of PML NBs per nucleus was also higher in HCC than in DN, LC and CH, which was not statistically significant (Table 1). Shape of PML NBs was quite different as well: greater than 50% of PML NBs in HCCs were large solid dots or ball-shaped with an empty core (Fig. 1C).

The average number of PML NB-positive HCC cells was greater in HCCs associated with HBV than in HCCs associated with HCV or alcohol (Table 2). The number of PML NBs per one tumor cell nucleus was also higher in HBV-associated HCCs than in HCV-associated HCCs or alcohol-associated HCCs (Table 2). The shape of PML NBs, howev-
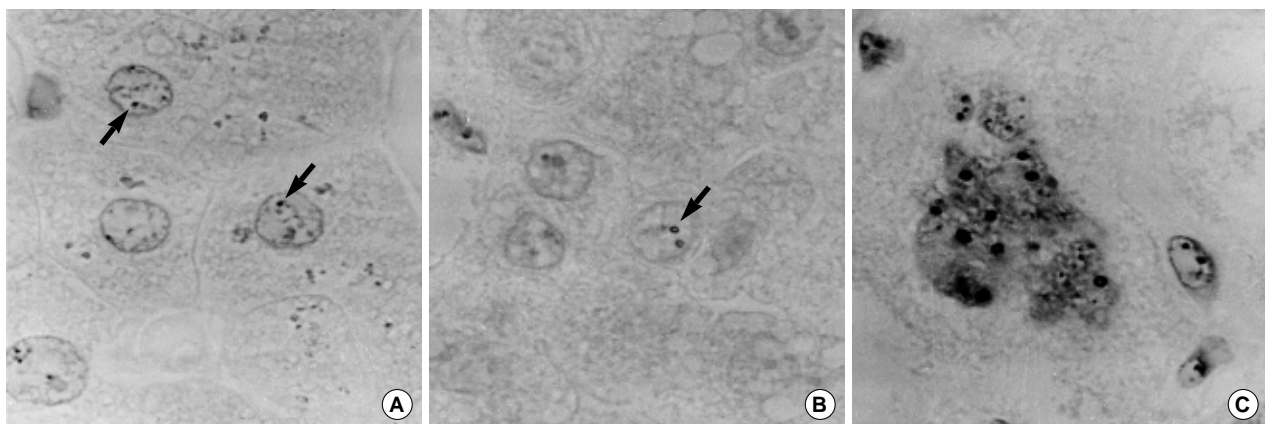

Fig. 1. PML expression in liver cirrhosis (A), dysplastic nodule (B), and hepatocellular carcinoma (C): A, Only one or two dots of PML NBs (arrows) are noted in regenerating hepatocytes. B, PML NBs are enlarged and some of them are ball-shaped (arrow). C, Most of PML NBs in HCC cells are markedly enlarged with empty cores (original magnification, ×1,000).
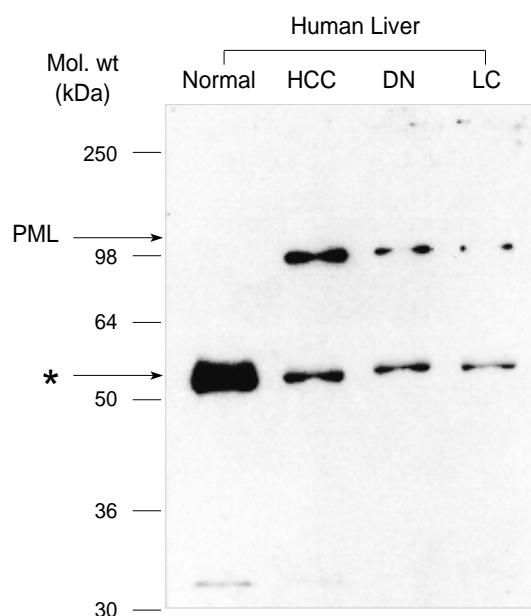
Fig. 2. Immunoprecipitation and immunoblot analysis for the PML in a case of hepatocellular carcinoma (HCC) with dysplastic nodule (DN) in a background of liver cirrhosis (LC). Note differential overexpression of the PML (arrow) in LC, DN and HCC. *, immunoglobulin heavy chain.
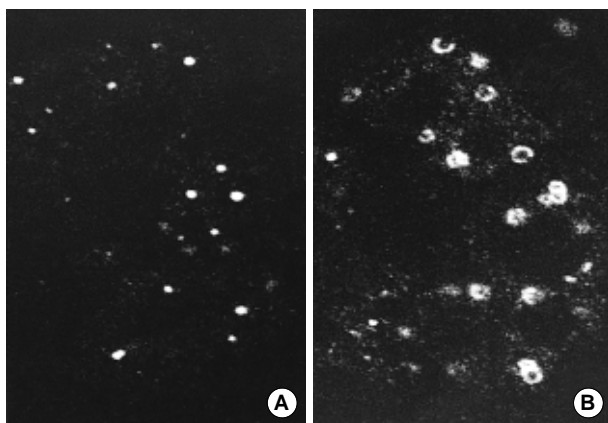

Fig. 3. PML NBs in HepG2 (A) and HepG2.2.15 cells (B) by confocal scanning microscope. Note enlarged ball-shaped PML NBs in HepG2.2.15 compared with a few fine dots in HepG2 cells (original magnification, ×1,000).

er, was similar in all HCCs. There was no significant correlation between the PML expression and histologic grade (Table 3). Pleomorphic nucleus in high grade HCCs, however, had greater number of much larger or ball-shaped PML NBs than in lower grade HCCs.

Differential overexpression of the PML in HCC compared with those in LC and DN was confirmed by immunoblotting in a case that had both DN and HCC in the background of LC (Fig. 2).

PML expression in hepatocellular carinoma cell lines

To examine the effect of HBV infection, we compared the expressions of PML in HepG2, HepG2.2.15, and Hep3B cells. HepG2.2.15 is a stable cell line that has been transfected by whole HBV genome in HepG2 cells and produce HBV DNA (20). HepG2 was established from a human hepatoblastoma and is not associated with HBV infection. Hep3B is a human hepatocellular carcinoma cell line producing HBV DNA and antigens. Five to ten PML NBs were detected in all HepG2 cells in interphase. Their shape was monotonous and about 0.5 μm in size (Fig. 3A). In HepG2.2.15 and Hep3B cells, the number of PML NBs was similar, but more than 50% of PML NBs were enlarged up to 1-2 μm with an empty core (Fig. 3B).

DISCUSSION

The development and progression of HCC is typical of a multistep process from chronic liver disease caused by viral infection or toxic substance through DN to early or advanced HCC with genetic and/or epigenetic alterations. Thus, we analyzed the PML expression in HCCs as well as DNs and adjacent non-neoplastic liver tissues in the present study. Although the PML is known to be present diffusely in the nucleoplasm or in PML NBs, the PML was localized mainly to PML NBs as revealed by immunohistochemical staining with the monoclonal antibody, PG-M3. Thus, we described the PML expression according to the number of PML NB-positive hepatocytes or HCC cells per 1,000 hepatocytes or HCC cells and the number of PML NBs in one nucleus of hepatocyte or HCC cell.

The differential overexpression of the PML in DN and HCC in this study indicates that the PML expression is differentially regulated during the multistep hepatocarcinogenesis, and the alteration of the PML expression may occur first in the early stage of hepatocarcinogenesis, from LC to DN formation. In contrast to our results, Chan et al. (15) described overexpression of the PML in the PML NBs in 50% of human HCCs and most of non-tumorous cirrhotic liver, thus indicated that the PML overexpression was associated with both LC and HCC formation. Gambacorta et al. (13) reported the PML expression in 10/10 liver tumors, but neither histologic grading of the HCCs nor comparative analysis between preneoplastic lesions and HCCs has been made. The immunoprecipitation with immunoblot analysis in a few representative cases of HCCs supported also a distinct overexpression of the PML in DN and HCC compared with non-neoplastic liver. To clarify a possibility of transcriptional upregulation, northern blot analysis using RT-PCR products of the PML in LC, DN and HCC has been undergoing. At this moment, however, it can not be determined whether the overexpression of the PML in DNs

and HCCs is a cause for hepatocellular carcinogenesis or an epiphenomenon during hepatocarcinogensis.

Statistically significant difference of the PML expression between HBV-associated HCCs and HCV- or alcohol-associated HCCs and morphological characteristics of PML NBs in HepG2.2.15, a stably transfected cell line by HBV genome, suggest a strong relationship between HBV infection and *PML* NBs. PML NBs are known to be the site of DNA virus transcription and replication (8), thus HBV infection may affect the size or shape of PML NBs. Aoki et al. (22) reported that a subgenomic HBV DNA sequence (15 AB) is a hot spot for genomic recombination and a portion of 15AB-like sequence is homologous to break-point clusters of the human *PML* gene. Considering a close relationship between HBV infection and HCCs, it may be suggested that the *PML* might be a recombinogenic candidate triggering genomic instability in HBV-associated hepatocarcinogenesis. The structure of ball-shaped PML NBs is known to be associated with a recruitment of other PML NB-associated proteins such as SUMO-1 (23, 24) and TRF1 or TRF2 (25), and assembly with PML within PML NBs. Thus a further analysis of coimmunoprecipitates with PML and PML isoforms in LC, DN and HCC using two-dimensional gel electrophoresis may elucidate an alternative mechanism of the PML overexpression and the morphological alteration of PML NBs.

ACKNOWLEDGMENTS

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