Decreased Expression of Phosphatase and Tensin Homolog Is Related with the Development of Non-B and Non-C Hepatocellular Carcinoma

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Objective: Recently, there has been a worldwide increase in non-B and non-C hepatocellular carcinoma (NBNC-HCC), which are negative for both markers of hepatitis B and C virus infection. Here, we investigated the role of phosphatase and tensin homolog (PTEN), a negative regulator of the phosphatidylinositol-3 kinase pathway in the development of NBNC-HCC.

Materials: A total of 14 patients who received hepatectomy because of NBNC-HCC (NBNC group) were analyzed retrospectively. Nine patients who underwent hepatectomy for liver metastasis of colorectal cancer, were analyzed as the control.

Methods: Expression of PTEN and p62, which is a marker of autophagic degradation, was evaluated in background liver, peritumoral region, and tumor by immunohistochemical analysis. Recurrence rate up to 7 years post-surgery was evaluated.

Results: The expression of PTEN in the NBNC group was significantly decreased in all regions compared to the control, and it was notably reduced in the tumor. In contrast, the expression of P62 in the tumor of patients of the NBNC group was significantly increased. Recurrence rate at 7 years in NBNC group reached 85%, and recurrence time post-surgery was significantly longer in survivors with strong expression of PTEN in the peritumoral region.

Conclusions: These findings indicate that the expression of PTEN decreases in NBNC chronic liver injury, which is possibly related to inhibition of autophagy as a trigger of carcinogenesis and the intrahepatic recurrence of NBNC-HCC.

Key words: autophagy, PTEN, p62, Non-B Non-C HCC, nonalcoholic steatohepatitis (NASH)

Introduction

The total number of hepatocellular carcinoma (HCC) cases in Japan peaked in the early 2000s due to the enlightened prevention activity of new infection cases of hepatitis and the progress of treatment for viral hepatitis, and it has been gradually decreasing. In contrast, the incidence of HCC without hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, so-called non-B non-C (NBNC) HCC, is rapidly increasing in Japan¹⁾ and also in many other developed countries²⁾. Although the exact cause underlying the increase of NBNC-HCC remains unclear, the involvement of increased

metabolic syndrome and non-alcoholic steatohepatitis (NASH) has been suggested¹⁾.

Phosphatase and tensin homolog (PTEN) is a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway, which contributes to cellular proliferative signals^{3) 4)}. Conditional hepatocyte-specific PTEN-knockout mice lead to spontaneous development of HCC, following hepatic steatosis, inflammation and fibrosis, thus resembling the features of NASH-HCC⁵⁾. Deficiency of PTEN also causes activation of mammalian target of rapamycin (mTOR), a resultant decrease in autophagic activity⁶⁾. However, the involvement of PTEN in human HCC is poorly understood.

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[Received Feb. 3, 2020] [Accepted Feb. 29, 2020]

J-STAGE Advance published date: Apr. 17, 2020

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doi: 10.14789/jmj.2020.66.JMJ20-OA01

Table-1 Characteristics of control and non-B non-C (NBNC) group

	Control (n=9)	NBNC (n=14)	p-value
Male/Female	7/2	12/2	1.000
Age	61 ± 2	69 ± 2*	0.016
AST (IU/l)	23 ± 3	27 ± 2	0.054
ALT (IU/l)	20 ± 3	25 ± 3*	0.046
γGTP (IU/l)	39 ± 8	115 ± 24**	0.003
T-Bil (mg/dl)	0.93 ± 0.09	0.77 ± 0.08	0.172
Plt (× 10 ⁴ μl)	22.5 ± 3.7	21.1 ± 1.6	0.382
PT (%)	91 ± 3	94 ± 2	0.115
FIB-4 index	1.76 ± 0.44	2.71 ± 0.59	0.095
Hb A1c	5.6 ± 0.3	5.7 ± 0.1	0.801
AFP (ng/ml, >20)	0/5 (0%)	4/14 (29%)	0.530
PIVKA II (mAU/ml, >40)	0/2 (0%)	13/14 (93%)*	0.025
Tumor diameter (mm)		53 ± 6	
Differentiation (Well/Moderate/Poor)		0/12/2	

PIVKAII; Protein induced vitamin K absence or antagonist II

*; p<0.05 vs control **; p<0.01 vs. control

Therefore, our aim in this study was to investigate the role of PTEN in development of NBNC-HCC.

Materials and Methods

1. Materials

A total of 14 patients who received hepatectomy because of hepatitis B virus surface antigen (HBsAg)-negative/hepatitis C virus antibody-negative HCC from March 2009 to February 2012 were analyzed retrospectively. Nine patients who received hepatectomy for liver metastasis of colorectal cancer were analyzed as the control group. Patient characteristics are shown in Table-1. Recurrence rate up to 7 years after surgery were evaluated.

This retrospective clinical study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Informed consent was obtained in the form of opt-out on the web-site (<https://www.gcprec.juntendo.ac.jp/kenkyu/detail/1793>) because of the retrospective design. This study was carried out with approval from the Hospital Ethics Committee Juntendo University Hospital (No. 19-082).

2. FIB-4 index

The FIB-4 index was calculated as follows: age

(yrs) × AST [U/l] / (platelets [10³/μl] × (ALT [U/l] 1/2)⁷).

3. Immunohistochemical staining

Tissue specimens from all patients and controls were fixed in formalin-fixed paraffin-embedded tissue. Immunohistochemical staining was performed using polyclonal antibody to PTEN (1:200, Cell Signaling Technology, Beverly, MA) and p62 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described⁸. Briefly, sections were cut 4 μm thick and were deparaffinized in xylene and dehydrated in descending dilutions of ethanol. For antigen retrieval, slides of PTEN were treated in citrate buffer (pH 6.0) and slides of p62 were treated in TE buffer (pH 9.0 DAKO, Carpinteria, CA) by boiling for 45 minutes. Endogenous peroxidase activity was blocked by 30 minutes of incubation with 0.3% hydrogen peroxide in absolute solution. The sections of tissue stained for PTEN and p62 were incubated with primary antibody, followed by the dextran polymer method (Chem Mate Envision; DAKO). Diaminobenzidine tetrahydrochloride was used to enhance the hydrogen peroxide stain and then, the sections were counterstained with Mayer hematoxylin.

4. Quantitative image analysis

Specimens for histology and immunohistochemistry

Figure-1 Definitions in this study

A: Definition of background (BG), period, tumor in this study. The peritumoral region was defined within an area of 5 mm from the tumor borders. **B:** Score of staining intensity of phosphatase and tensin homolog (PTEN) or p62 in liver or tumor tissue.

were observed under an optical microscope (PH-2; Olympus, Tokyo, Japan) equipped with a digital microscope camera (VB6000; Keyence, Osaka, Japan). The intensity of positive staining was assessed separately for background liver (BG), peritumor and tumor region. The peritumoral region was defined within an area of 5 mm from the tumor borders. The expression of PTEN was evaluated based on the intensity of cytoplasmic staining, and the expression of p62 was evaluated based on the frequency of p62-positive inclusion bodies in the cytoplasm, each of which was scored 0 to 3 representing negative, weak, moderate, and strong, respectively (Figure-1).

5. Statistical analysis

Data were expressed as means $\pm$ SEM. Comparisons among 3 groups of quantitative and qualitative variables were performed using analysis of variance (ANOVA) on ranks, followed by an all pairwise multiple comparison procedure (Student-Newman-Keuls Method), and Mann-Whitney U-test was performed to evaluate the relationship between 2

groups as appropriate. The Fisher's Exact Test was used to examine gender differences and positive ratio of serum alpha-fetoprotein (AFP) and protein-induced vitamin K absence, or antagonist II (PIVKA II). $p < 0.05$ was selected before the study to reflect significance.

Results

1. Clinical background of patients with NBNC-HCC

Age and serum ALT and γ -glutamyl transpeptidase (γ GTP) levels in NBNC group were significantly higher than those in the control group. The positive rate of serum levels of PIVKA II was as high as 93% in the NBNC group, while that of AFP was as low as 29%. Although the average value of FIB-4 index was also higher in the NBNC group than in the control group, there was no significant statistical difference. The tumor diameter of HCC was as large as 53 ± 6 mm, and the tumor differentiation was moderate in all cases except for two cases with poorly differentiated HCC (Table-1).

Figure-2 Expression of PTEN in the livers with NBNC-HCC is generally reduced, particularly intra tumor. Average score of PTEN staining intensity were plotted. A: Changes in PTEN expression from the background liver to the tumor region. *: vs. background (BG), #: vs. peritumor, $p < 0.05$ by ANOVA on ranks and Student–Neuman–Keuls post-hoc test. B: Region-by-region comparison of PTEN expression between control and NBNC group. *: vs. control, $p < 0.05$, ***: vs. vs. control, $p < 0.001$, by Mann–Whitney U-test. Error bars represent mean $\pm$ SEM.

2. Patterns of PTEN and p62 expression in NBNC-HCC

Immunohistochemical staining indicated that PTEN expression was localized diffusely in the cytoplasm of parenchyma cells. On the contrary, p62 immunostains showed granular cytoplasmic staining (Figure-1). While the expression of PTEN in the control group was not significantly different among BG, peritumor and tumor, the average expression of PTEN in the NBNC group decreased in the order of BG, peritumor, and tumor, with the expression of PTEN in the tumor region being significantly lower compared to that in BG and peritumor (Figure-2A). When the expression of PTEN was compared according to the positional relationship with that of tumor, PTEN expression in NBNC group was significantly lower than what was found in the control group of BG, peritumor and tumor regions. The expression of PTEN in the tumoral region was particularly reduced in the NBNC group compared to that in the control group (Figure-2B).

The expression of p62 was significantly increased in the tumor region of the NBNC group, whereas little to no expression was observed in tumor of the control group (Figure-3A). When the expression of p62 was compared between the control group and the NBNC group according to the positional relationship with the tumor, there was no difference between the control and the NBNC group in BG and peritumor. On the contrary, the expression of p62 in tumor was markedly different between the control group and the NBNC group (Figure-3B).

3. Relationship between PTEN/p62 expression and recurrence-free survival in NBNC-HCC

The recurrence-free survival rate dropped rapidly until 115 weeks post-surgery, reaching 30.1% following a gradual and continued decrease to 15.0% at 5.4 years (Figure-4). Analysis of the relationship between PTEN expression and recurrence-free period indicated that the group with high PTEN expression in peritumor had a significantly longer recurrence-free period (Figure-5A,

Figure-3 Expression of p62 in the livers with NBNC-HCC is increased intra tumor. Average score of p62 staining intensity were plotted. A: Changes in p62 expression from the background liver to the tumor region. *: vs. background (BG), #: vs. peritumor, $p < 0.05$ by ANOVA on ranks and Student-Neuman-Keuls post-hoc test. B: Region-by-region comparison of p62 expression between control and NBNC group. **: vs. control, $p < 0.01$, by Mann-Whitney U-test. Error bars represent mean $\pm$ SEM.

$p < 0.05$). On the contrary, there was no marked tendency between the expression of p62 and the recurrence-free period (Figure-5B).

Discussion

Serum ALT and γ GTP levels were significantly higher in NBNC group compared to control group, which indicated liver injury in background livers. On the contrary, the fibrosis marker FIB-4 index in NBNC did not reach a significant difference from the control, although it was generally higher than the control. In a previous study, FIB-4 2.87 or more was reported as a significant value suggesting moderate-to-severe liver fibrosis⁹⁾, which is higher than the average of the NBNC group this present study. FIB-4 in the NBNC group did not reach a significant level possibly because the subjects of this study being biased to patients keeping liver function because they underwent hepatectomy. Moreover, NBNC-HCC could develop without advanced fibrosis. Indeed, NBNC-HCC has been reported to develop in a less advanced state of

Figure-4 Recurrence free period after hepatic surgery

Figure-5 Decreased expression of peritumoral PTEN is associated with postoperative recurrence of NBNC-HCC

The number of weeks until recurrence of HCC after liver surgery was plotted for each score of PTEN (A) or p62 (B) expression in BG, peritumor and the tumor region. *: vs. score 0-1, $p < 0.05$ by ANOVA on ranks and Student-Neuman-Keuls post-hoc test. Error bars represent mean $\pm$ SEM. The boxes represent the mean number of weeks and the line in the box represents median. The bars across the boxes show the minimum and maximum weeks. Data represent mean $\pm$ SD.

background liver fibrosis compared to HCV-HCC¹⁰⁾. The sensitivity of PIVKAI in the NBNC group was much higher than that of AFP. It has been reported that the sensitivity of PIVKAI is almost the same value as that of AFP in viral hepatitis-related HCC, and it was higher than that of AFP in NBNC-HCC¹¹⁾, which is also consistent with our findings (Table-1).

In the present study, the expression of PTEN was markedly reduced in the tumor region in NBNC group. In note, comparing with the control group, the expression of PTEN was decreased in all regions including the background liver and peritumor in the NBNC group (Figure-2). These findings indicate that the reduction of PTEN in hepatocytes occurs in the molecular background of NBNC-HCC patients, resulting in further reduction of PTEN as an initiating step of liver carcinogenesis. Deficiency of PTEN is considered to be involved in tumor growth because it is a negative regulator of

PI3K^{4) 12)}. However, regulation of PTEN seems to be involved in multiple ways in the pathogenesis of non-viral liver diseases, particularly steatohepatitis. In liver-specific PTEN knockout mice, expression of SREBP-1c in the liver is increased, and *de novo* lipogenesis is activated to progress hepatic steatosis⁵⁾. Additionally, PTEN is involved in inhibiting hepatic stellate cell activation, which is critical in hepatic fibrogenesis¹³⁾. Although the involvement of steatohepatitis such as NASH and alcoholic liver disease (ALD) in NBNC-hepatic carcinogenesis has been suspected, it is often difficult to clinically clarify the involvement of hepatic steatosis in hepatic carcinogenesis because fat deposition is lost through the progression of NASH (burned-out NASH)¹⁴⁾. ALD also burns out as the disease progresses¹⁵⁾, making it difficult to definitively diagnose the etiology if there is no history of a prior consultation. Since the majority of patients in the NBNC group of this study came to

our hospital after detection of liver cancer, the clinical course pre-hospitalization is unclear, and the percent occupied by ALD or NASH is unknown. According to the national tabulation conducted by the Japan Society of Hepatology in 2011, 14.5% of NBNC-cirrhosis was based on NASH and 55.1% was based on ALD¹⁶⁾. Therefore, it is assumed that NBNC group in the current study also include many cases of NASH and ALD. Taken together, it is suggested that the reduction of PTEN in NBNC-HCC is involved in the pathogenesis of nonalcoholic and alcoholic steatohepatitis and the development/progression of HCC.

Autophagy is a self-degradative process in which cytoplasmic components including organelles are sequestered by double membrane structures called autophagosomes and sequestered materials are degraded by lysosomal hydrolase¹⁷⁾. Although autophagy is induced by starvation and has been regarded as a means of nutrient supply during malnutrition, it has recently become clear that autophagy plays a crucial role in maintaining intracellular homeostasis by periodically replacing old organelles with new ones, post-degradation¹⁸⁾. Additionally, autophagy activation has been reported to contribute to the suppression of tumorigenesis^{19) 20)}. p62 is the target protein for autophagy and in this study its expression was evaluated in parenchymal cells of NBNC-HCC patients. The expression of p62 in the NBNC group was significantly enhanced in the tumor region when compared to BG and peritumor (Figure-3). This finding suggests that the degradation after autophagosome formation in steps of autophagy is suppressed in the NBNC-HCC. We have previously reported that autophagy activity is reduced in the fatty liver in mice and NASH in humans^{8) 18)}, and the result in this study also suggested that suppression of autophagy could possibly contribute to NBNC liver carcinogenesis. The PI3K pathway activated by PTEN deficiency activates mammalian target of rapamycin (mTOR), a central regulator of autophagy^{21) 22)}. The enhanced expression of p62 is consistent with the result of the suppression of autophagy due to the decrease in PTEN expression in the tumor region.

The tumor diameter as large as average 53 mm and the absence of well-differentiated HCC are possibly the causes of the increased recurrence rate

of NBNC-HCC in this study (Table-1). Alternatively, only one case in NBNC group showed portal vein invasion, suggesting that most of the recurrent cases in the present study were based on multicentric carcinogenesis although it is difficult to be distinguished strictly. Anyway, in this NBNC-HCC group, post-surgery recurrences gradually increase, which shows the difficulty of preventing HCC-recurrence without suppression of the underlying molecular mechanism in liver carcinogenesis (Figure-4). In addition, maintaining high expression of PTEN in peritumor was shown to be a key factor for suppression of HCC recurrence post-surgery (Figure-5). This result also suggests that the reduction of PTEN expression in hepatic parenchymal cells is the final trigger of hepatic carcinogenesis.

To clarify the clinical significance of these findings, confirmatory studies should be performed because this study is based on the pathological evaluation of just a limited part of the whole tumor, so it is possible that the evaluation is biased by the heterogeneity of PTEN or p62 expression. In the future, however, it is expected that the therapeutic intervention should maintain PTEN expression and contribute to the prevention of recurrence of NBNC-HCC after radical treatment.

In conclusion, our findings imply that step-wise reduction of PTEN in hepatocytes is a trigger of carcinogenesis and the intrahepatic recurrence in NBNC-HCC and disruption of autophagy is closely related to the progression of NBNC-HCC. It was suggested that modulation of the PTEN-autophagy axis is involved in hepato-carcinogenesis, and the regulation of the process is a promising novel target for preventing the development/recurrence of NBNC HCC.

Funding

The authors received no financial support for the research.

Authors' contribution

YS and KK designed and performed the experiments. HS performed the histological examination of the liver tissue. AU, KF, and SH collected clinical data. AA and KI verified the analytical methods. All authors discussed the results and contributed to the final manuscript.

Conflict of interest

The Authors' declare that there is no conflict of interest.

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