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INCREASED EXPRESSION OF TOLL-LIKE RECEPTOR 4 IN HEPATOCELLULAR CARCINOMA

—— ITS CLINICOPATHOLOGICAL SIGNIFICANCE ——

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Abstract
Lipopolysaccharide (LPS), known as endotoxin, is produced by Gram-negative bacteria and transported from the intestine to the liver via the portal vein. While LPS is cytotoxic and gives damages to hepatocytes, it also functions as an exogenous ligand for toll-like receptor 4 (TLR4) expressed in the plasma membrane. Stimulated by LPS, TLR4 activates MyD88-NF-κB signaling pathway in hepatocytes, leading to tissue repair and cell proliferation. It has thus been postulated that a combination of LPS-induced hepatic injury and TLR4-mediated tissue repair may lead to development of hepatocellular carcinoma (HCC). As recently reported, the increased expression of TLR4 in HCC is related to poor prognosis, suggesting involvement of TLR4 in HCC progression after its development. To explore correlation of clinicopathological characteristics of HCC with TLR4 expression, we immunostained TLR4 protein in both HCC and its neighboring non-cancerous area by using 99 resected livers bearing HCC. In general, TLR4 expression was significantly higher in HCC areas than in non-cancerous areas. When correlation of the increased expression of TLR4 with different clinicopathological factors was assessed, tumor size and expanding growth pattern were significantly correlated with the TLR4 expression level, suggesting that TLR4 is closely related to cell proliferation but not invasive phenotype.

Key words : toll-like receptor 4, lipopolysaccharide, hepatocellular carcinoma, surgical specimen, immunohistochemistry

Introduction

While the gastrointestinal tract always encounters foreign bodies called "food," the liver is not exposed directly to extracorporeal environment. There are two paths connecting between the gastrointestinal tract and the liver. One is the biliary tract for discharge of bile from the liver to the intestine, the other is the portal vein for transportation of nutrients from the intestine to the liver. The latter serves as not only a supply route of nutrients but also an intake of various toxic materials, including lipopolysacharride (LPS) produced by Gram-negative bacteria in intestinal microbiota[1].

LPS is a component of Gram-negative bacterial cell wall and functions as endotoxin[1]. When composition of intestinal microbiota has aberrantly altered, when Gram-negative bacteria proliferate excessively in the intestine,

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or when barrier function of the intestinal epithelium is impaired, the amount of LPS transported to the liver via the portal vein drastically increases[2]. It is well known that LPS has strong cytotoxicity causing direct damage to hepatocytes. LPS can also insult the liver tissue by means of indirect mechanisms, *i.e.*, LPS activates an apoptotic pathway in hepatocytes by inducing Kupffer cells to secrete TNF-α[2].

On the other hand, receptor molecules, called toll-like receptor 4 (TLR4), specific for LPS are expressed on cell membrane of the hepatocytes. TLR4 protein activated by LPS contributes to tissue repair, regeneration, and proliferation by involving a MyD88-NF-κB signaling pathway[1,3-5]. It has, thus, been postulated that a combination of LPS-induced inflammatory hepatic injury and TLR4-mediated tissue repair and/or proliferation may lead to development of hepatocellular carcinoma (HCC). There is indeed a growing body of evidence indicating that TLR4 should enhance tumor promotion stage during hepatocarcinogenesis before HCCs have developed[3,6-9]. However, it has recently been reported that the expression level of TLR4 protein in HCC is in reverse correlation with survival of the patients, suggesting that TLR4 should be involved in not only carcinogenesis but also progression of developed HCC[10]. If it is the case, what kind of clinicopathological characteristics of HCC is related to TLR4 expression? To address the question, we therefore examined expression patterns, as revealed by immunohistochemistry, of TLR4 protein in both HCC and its neighboring non-cancerous area and analyzed the extent of correlation between each clinicopathological factor and the increased expression of TLR4 in HCC.

Materials and Methods

Tissue specimens

All the samples examined for the present study were formalin-fixed paraffin-embedded (FFPE) sections generated from HCCs and their neighboring non-cancerous liver tissues resected from 99 patients operated in Akita University Hospital in the period from 2005 to 2012. All the samples were diagnosed by experienced pathologists. For experiments, well fixated FFPE blocks containing a minimal size of necrotized area were selected and 3-μm-thick slices were subjected to immunohistochemistry as well as hematoxylin-eosin (HE) staining. We obtained informed consent from all patients before their enrollment in this study. The study was conducted in accordance with the Declaration of Helsinki.

Immunohistochemistry

The mouse anti-TLR4 monoclonal antibody (clone 76B357.1, Abcam, Cambridge, UK) was purchased. FFPE sections on slide glass were deparaffinized, autoclaved in 1 mM EDTA pH 9.0 at 121°C for 20 min, then immersed in 3% hydrogen peroxide/methanol at room temperature for 15 min. The slides were incubated with the primary antibody in a humidified chamber at 4°C overnight (17 h). Specific signals were visualized by employing HRP-labeled polymer method as follows, the slides were reacted with EnVision+ system-HRP for mouse (Agilent, Santa Clara, CA) at room temperature for 30 minutes and finally DAB was oxidized for signal detection.

Image analysis and quantification

Positive intensity of immunohistochemical signals for TLR4 protein was quantified and analyzed using ImageJ 1.51 j8. The microscope capture images were converted to 8 bit gray scale and luminance displayed in the histogram was recorded. Therefore, a stronger DAB staining indicates a lower numerical value, whereas a weaker one indicates a higher numerical value. To avoid effects of non-specific background staining, ratio of each raw value to that of the area where TLR4 should not be expressed was gotten, and finally this ratio was subtracted from 1 to get the positive index. All the analyses were performed based on these positive indices.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 24.

Results

Clinicopathological characteristics

Clinicopathological profiles of all the cases examined for the present study are summarized in Table 1. 99 pa-

Table 1. Clinicopathological characteristics (n=99)

Gender		
	Male	81.8% (81/99)
	Female	18.2% (18/99)
Age (years)		
	Max	80
	Min	40
	median	67
	mean±SD	65.98±10.07
Hepatitis virus Infection (duplicate=1)		
	HBs Ag positive	19.2% (19/99)
	HCV Ab positive	41.4% (41/99)
	otherwise	40.4% (39/99)
Tumor size/diameter at the maximum equator (cm)		
	Max	24.0
	Min	0.8
	median	4.5
	mean±SD	6.02±4.4
Tokyo score		
	0	4.0% (4/99)
	1	51.5% (51/99)
	2	44.4% (44/99)
Histological grading (Edmondson grade)		
	I	7.1% (7/99)
	II	49.5% (49/99)
	III	39.4% (39/99)
	IV	3.0% (3/99)
	unknown	1.0% (1/99)
Staging (T)		
	T1	5.1% (5/99)
	T2	36.4% (36/99)
	T3	34.3% (34/99)
	T4	21.2% (21/99)
	unknown	3.0% (3/99)
Growth pattern		
	eg	44.4% (44/99)
	eg > ig	39.4% (39/99)
	ig > eg	12.1% (12/99)
	ig	4.0% (4/99)

tients, who had their HCCs surgically-resected in Akita University Hospital in the period from 2005 to 2012, are subjected to our study. The males account for 81.8% (81/99 patients). The ages are ranged from 40 to 80 years old (median 67). As for the status of hepatitis viral infection, 19.2% (19/99) and 41.4% (41/99) are positive for HBs Ag and HCV Ab, respectively. The tumor sizes are ranged from 0.8 to 24.0 cm (median 4.5). Applied to Tokyo score (0 to 2)[11], 51.5% and 44.4% are scored 1 and 2, respectively. According to Edmondson

grading[12,13], 50.0% and 39.8% are evaluated as Grade II and III, respectively, while only a few cases are evaluated as Grade I or IV. Tumor staging designated by the T factor is, in order of high proportion, T2 (37.5%) > T3 (35.4%) > T4 (21.9%) > T1 (5.2%)[14]. 83.8% of our tumor collection exhibits an expanding growth (eg) pattern (eg, 44.4% ; eg>infitrating growth (ig) pattern, 39.4%).

Expression patterns of TLR4 protein in the livers bearing HCC

Firstly, we examined expression patterns of TLR4 protein in the livers bearing HCC from 99 patients. In all the cases examined, TLR4 is expressed, as revealed by immunohistochemistry, most strongly in cholangiocytes, notably in those showing ductular reaction which often occurs in a non-cancerous area adjacent to an HCC lesion (Fig. 1). Intense signals of TLR4 are also observed diffusely in cell membrane and cytoplasm of both HCC cells and non-cancerous hepatocytes (Fig. 1). Increased expression of TLR4 in hepatocytes has been known to elevate susceptibility to development of HCC[6]. Our data thus revealed that expression of TLR4 protein was sustained even after HCC had developed.

Comparison of expression levels of TLR4 between HCC and non-cancerous areas

As a clue to understand importance of TLR4 expression in the developed HCCs, we carefully compared the intensity of immunohistochemical signals for TLR4 between HCC and non-cancerous areas in the same liver of each case. The intensities were measured on captured images by using ImageJ software and each acquired value was converted to a positive index as described in Materials and Methods section. The indices of TLR4 expression both in HCC areas and in non-cancerous areas showed a normal distribution statistically. As shown in Table 2 and Fig. 2, TLR4 expression is significantly higher in HCC areas than in non-cancerous areas ($p<0.0001$).

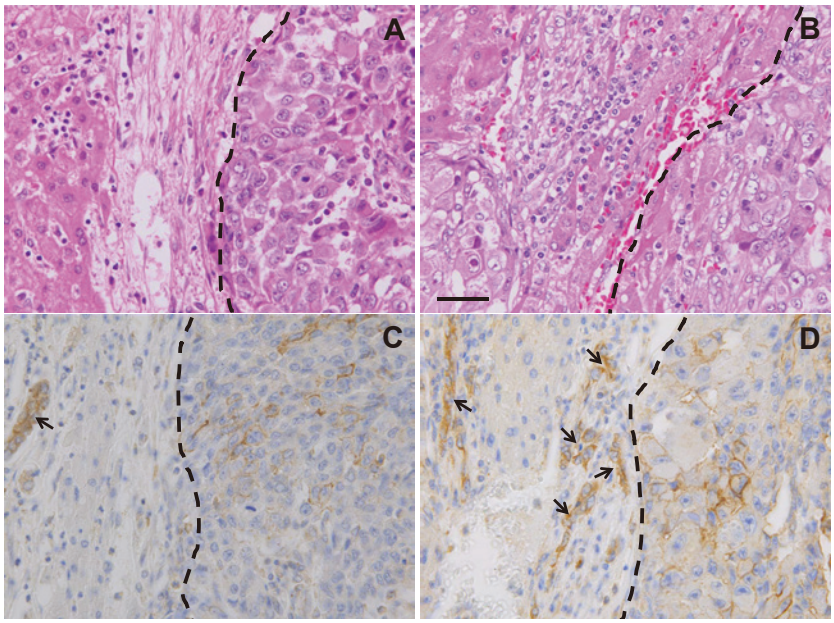

Fig. 1. Immunostaining of TLR4 in the liver bearing HCC. Two representative cases are featured. (A) and (B) are HE staining. (C) and (D) are the images of immunohistochemistry, captured from the sites almost corresponding to (A) and (B), respectively, on each serial section. In each micrograph, HCC is presented in the right to the dotted line. Non-cancerous hepatocytes are presented in the left of each image with the central zone intervened by connective tissue. Biliary ducts and/or ductules are highlighted by arrows. Scale bar, 50 μm.

Table 2. TLR4 index significant difference test

		n	Tumor area	non Tumor area	*p* value
			Mean±SD	Mean±SD	
	Total	99	0.28±0.11	0.24±0.09	<0.0001
Tokyo Score					
	0	4	0.21±0.07	0.20±0.47	0.391
	1	51	0.30±0.09	0.26±0.09	<0.0001
	2	44	0.26±0.12	0.23±0.10	<0.0001
Edmondson Grade					
	I	7	0.20±0.08	0.19±0.42	0.731
	II	49	0.29±0.12	0.25±0.10	0.029
	III	39	0.29±0.10	0.24±0.09	0.004
	IV	3	0.27±0.05	0.21±0.21	0.435
Staging (T)					
	T1	5	0.21±0.10	0.19±2.00	0.521
	T2	36	0.27±0.12	0.25±0.77	0.180
	T3	34	0.31±0.99	0.24±0.79	<0.0001
	T4	21	0.28±0.09	0.25±0.97	0.248
Hepatitis virus Infection (duplicate=1)					
	HBs Ag positive	19	0.28±0.13	0.24±0.07	0.120
	anti-HCV Ab positive	41	0.30±0.30	0.25±0.06	<0.001
	otherwise	40	0.26±0.11	0.24±0.11	0.105
Growth pattern of tumor					
	eg	44	0.29±0.12	0.26±0.10	0.062
	eg > ig	39	0.29±0.09	0.24±0.08	<0.001
	ig > eg	12	0.21±0.10	0.19±0.11	0.382
	ig	4	0.27±0.13	0.22±0.04	0.437

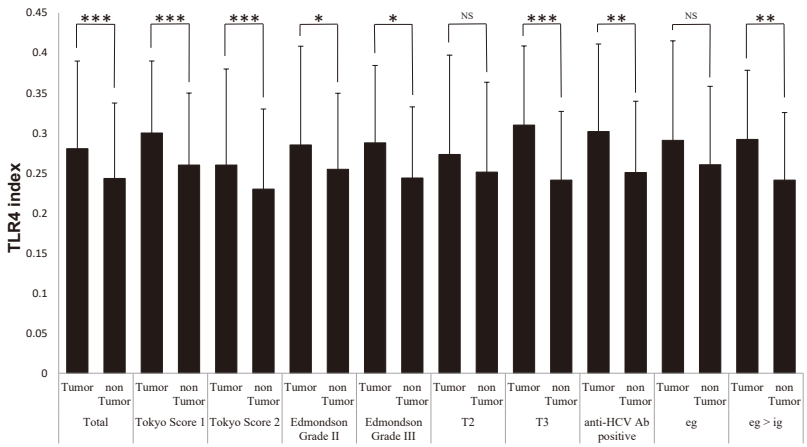

Fig. 2. Correlation of the increased expression of TLR4 with different clinicopathological characteristics. Only the results showing significant correlation in Table 2 are demonstrated. TLR4 expression index was calculated as described in Materials and Methods section. The measurable maximal index should be 1.
*, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$; NS, not significant. Error bar represents standard deviation.

Elevated expression of TLR4 in HCC according to various clinicopathological factors

It has been reported that the expression level of TLR4 is in inverse correlation with prognosis of HCC patients[10]. To explore which clinicopathological factors of HCC can be modulated by TLR4 expression, we further performed paired-sample *t*-tests on the TLR4 expression level between HCC and non-cancerous areas according to various clinicopathological factors including gender, Tokyo score (0 to 2), histological grade (Edmondson I to IV), staging parameter (T1 to T4), growth pattern (eg or ig), and status of hepatitis virus infection. As shown in Table 2 and Fig. 2, regardless of genders, Tokyo scores, or histological grades, TLR4 protein is expressed more intensely in HCC areas than in non-cancerous areas, thus suggesting no or insignificant involvement of these clinicopathological factors in the elevated expression of TLR4 protein in HCCs. In contrast, our data have indicated that the tumor staging parameter, tumor growth pattern, and status of hepatitis virus infection are related to the enhanced expression of TLR4 in HCC. It is in tumors categorized into Edmondson II and III but not into I or IV that TLR4 expression levels are significantly higher than in non-cancerous areas. While HCCs exhibiting predominantly an expanding growth pattern, *i.e.*, tumors judged as "eg" or "eg>ig," give significantly stronger signals of TLR4 protein than non-cancerous areas, those exhibiting predominantly an infiltrating growth pattern ("ig" or "ig>eg") express insignificantly different levels of TLR4 protein from non-cancerous areas. As for the status of hepatitis virus, HCV-related HCCs but not HBV-related ones express a significantly larger amount of TLR4 protein compared with non-cancerous areas.

Discussion

NF-κB-mediated TLR4 signaling initiated by binding of LPS to its receptor TLR4 plays pivotal roles in hepatocarcinogenesis[7,15]. In the present study, we however found out that, even after cancer development, HCC lesions expressed a larger amount of TLR4 protein compared with hepatocytes in non-cancerous areas in the same liver. Furthermore, expression of TLR4 in HCC has recently been proposed to be one of signs for poor prognosis[10]. To understand how TLR4 contributes to deterioration of HCC patients' survival, we assessed association of TLR4 expression patterns with different clinicopathological factors. Among the clinicophathological findings verified, five factors such as Tokyo score, histological grade, staging parameter, growth pattern, and status of hepatitis virus infection showed significant correlation with increased expression of TLR4 in HCC (Table 2 and Fig. 2). Tumor staging T2 and T3 as well as Tokyo score are the indices based principally on tumor size[11,14], while T4 tends to be influenced by the factor of vascular invasion. Our results thus suggest that TLR4 may have potential to up-regulate proliferation of HCC cells rather than invasion, resulting in significant correlation of TLR4 expression with staging T2 and T3. Consistently, correlation of expanding growth pattern, judged as "eg" or "eg>ig," with TLR4 expression is also significant, suggesting that TLR4 expression may enable HCC cells to escape from contact inhibition without impairing cell-cell adhesion. On the other hand, infiltrating growth pattern, resulting from loss of cell-cell adhesion, seems to be independent of TLR4.

LPS used to be believed to behave as a negative regulator of cancer due to its ability to induce anti-tumor T-cell response in a TLR4-mediated manner[3,16]. This perception is still valid. However, it is also true that recent studies have revealed carcinogenic roles of LPS-stimulated TLR4[3]. It seems that roles of TLR4 in cancer development vary depending on organs and phases of carcinogenesis[17-20]. Amongst all the internal organs, the liver is the organ which encounters the highest density of LPS in an innate and physiological condition. The liver may thus be programmed to develop an efficient protective response against LPS-induced cytotoxicity, resulting in predominance of the oncogenic effect of LPS.

Although LPS-induced liver injury and TLR4 signaling are implicated in hepatocarcinogenesis, the present study has indicated that TLR4 signaling also contributes to progression of HCC, in other words, that LPS transported from the intestine to the liver can enhance progression of HCC. Therefore, improving the quality of the intestinal microbiota leads to both prevention of HCC and improve-

ment of its prognosis[21]. These aspects should be further verified carefully through experimental and clinical studies.

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References

1) Lu, Y.C., Yeh, W.C. and Ohashi, P.S. (2008) LPS/TLR4 signal transduction pathway. *Cytokine*, **42**, 145–151.
2) Bawa, M. and Saraswat, V.A. (2013) Gut–liver axis : role of inflammasomes. *J. Clin. Exp. Hepatol*., **3**, 141–149.
3) Rakoff-Nahoum, S. and Medzhitov, R. (2009) Toll-like receptors and cancer. *Nat. Rev. Cancer*, **9**, 57–63.
4) Takeda, K. and Akira, S. (2004) TLR signaling pathways. *Semin. Immunol*., **16**, 3–9.
5) Vaquero, J., Campbell, J.S., Haque, J., McMahan, R.S., Riehle, K.J., Bauer, R.L. and Fausto, N. (2011) Toll-like receptor 4 and myeloid differentiation factor 88 provide mechanistic insights into the cause and effects of interleukin-6 activation in mouse liver regeneration. *Hepatology*, **54**, 597–608.
6) Dapito, D.H., Mencin, A., Gwak, G.Y., *et al.* (2012) Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell*, **21**, 504–516.
7) Maeda, S. (2010) NF-kappaB, JNK, and TLR Signaling Pathways in Hepatocarcinogenesis. *Gastroenterol. Res. Pract*., **2010**, 367694.
8) Seki, E., Park, E. and Fujimoto, J. (2011) Toll-like receptor signaling in liver regeneration, fibrosis and carcinogenesis. *Hepatol. Res.*, **41**, 597–610.
9) Yang, L., Xu, X.G., Zhang, J.G., Xu, Q.R. and Huang, D.S. (2016) Accumulating evidence : TLR signaling facilitates the development of hepatocellular carcinoma. *Int. J. Clin. Exp. Med*., **9**, 4107–4112.
10) Eiro, N., Altadill, A., Juarez, L.M., *et al.* (2014) Toll-like receptors 3, 4 and 9 in hepatocellular carcinoma : Relationship with clinicopathological characteristics and prognosis. *Hepatol. Res*., **44**, 769–778.
11) Tateishi, R., Yoshida, H., Shiina, S., *et al.* (2005) Proposal of a new prognostic model for hepatocellular carcinoma : an analysis of 403 patients. *Gut*, **54**, 419–425.
12) Edmondson, H.A. and Steiner, P.E. (1954) Primary carcinoma of the liver : a study of 100 cases among 48,900 necropsies. *Cancer*, **7**, 462–503.
13) Schlageter, M., Terracciano, L.M., D'Angelo, S. and Sorrentino, P. (2014) Histopathology of hepatocellular carcinoma. *World J. Gastroenterol*., **20**, 15955–15964.
14) Brierley, J.D., Gospodarowicz, M.K. and Wittekind, C. (2017) *TNM classification of malignant tumours, Eighth Edition*. John Wiley & Sons, Oxford, UK.
15) Wang, L., Zhu, R., Huang, Z., Li, H. and Zhu, H. (2013) Lipopolysaccharide-induced toll-like receptor 4 signaling in cancer cells promotes cell survival and proliferation in hepatocellular carcinoma. *Dig. Dis. Sci*., **58**, 2223–2236.
16) Apetoh, L., Ghiringhelli, F., Tesniere, A., *et al.* (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat. Med*., **13**, 1050–1059.
17) Garin, A., Meyer-Hermann, M., Contie, M., Figge, M.T., Buatois, V., Gunzer, M., Toellner, K.M., Elson, G. and Kosco-Vilbois, M.H. (2010) Toll-like receptor 4 signaling by follicular dendritic cells is pivotal for germinal center onset and affinity maturation. *Immunity*, **33**, 84–95.
18) Kanczkowski, W., Tymoszuk, P., Ehrhart-Bornstein, M., Wirth, M.P., Zacharowski, K. and Bornstein, S.R. (2010) Abrogation of TLR4 and CD14 expression and signaling in human adrenocortical tumors. *J. Clin. Endocrinol. Metab*., **95**, E421–429.
19) Kelly, M.G., Alvero, A.B., Chen, R., Silasi, D.A., Abrahams, V.M., Chan, S., Visintin, I., Rutherford, T. and Mor, G. (2006) TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. *Cancer Res*., **66**, 3859–3868.
20) Sato, Y., Goto, Y., Narita, N. and Hoon, D.S. (2009) Cancer Cells Expressing Toll-like Receptors and the Tumor Microenvironment. *Cancer Microenviron*., **2 Suppl 1**, 205–214.
21) Darnaud, M., Faivre, J. and Moniaux, N. (2013) Targeting gut flora to prevent progression of hepatocellular carcinoma. *J. Hepatol*., **58**, 385–387.