Expression of TLR-2 in hepatocellular carcinoma is associated with tumour proliferation, angiogenesis and Caspase-3 expression

Running Title: Expression of TLR2 in hepatocellular carcinoma

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Abbreviations

HCC: Hepatocellular carcinoma

TLRs: Toll like receptors

VEGF: Vascular endothelial growth factor

Abstract

Aims: Unlike other Toll-like receptors (TLRs), the role of toll like receptor 2 (TLR-2) in the pathogenesis of chronic liver disease and hepatocellular carcinoma (HCC) is not well studied. We, therefore, set out to investigate the expression of TLR-2 in different chronic liver disease states along with other markers of cell death, cellular proliferation and tissue vascularisation

Methods and results: Immunohistochemistry was performed on liver tissue microarrays comprising hepatitis, cirrhosis and HCC patient samples using antibodies against TLR-2, Ki-67, Caspase-3 and VEGF. This was done in order to characterise receptor expression and translocation, apoptosis, cell proliferation and vascularisation. Cytoplasmic TLR-2 expression was found to be weak in 5/8 normal liver cases, 10/19 hepatitis cases and 8/21 cirrhosis patients. Moderate to strong TLR-2 expression was observed in some cases of hepatitis and cirrhosis. Both, nuclear and cytoplasmic TLR-2 expression was present in HCC with weak intensity in 11/41 cases, and moderate to strong staining in 19/41 cases. Eleven HCC cases were TLR-2 negative. Surprisingly, both cytoplasmic and nuclear TLR-2 expression in HCC were found to significantly correlate with proliferative index (r=0.24 and 0.37), Caspase-3 expression (r=0.27 and 0.38) and vascularisation (r=0.56 and 0.23). Further, nuclear TLR-2 localisation was predominant in HCC, whereas cytoplasmic expression was more prevalent in hepatitis and cirrhosis. Functionally, treatment of HUH7 HCC cells with a TLR-2 agonist induced the expression of cellular proliferation and vascularisation markers CD34 and VEGF.

Conclusions: Our results demonstrate a positive correlation between the expression of TLR-2 and other markers of proliferation and vascularisation in HCC which suggests a possible role for TLR2 in HCC pathogenesis

Key words: TLR2; HCC; proliferation; vascularisation

Introduction

More than two-thirds of reported hepatocellular carcinoma (HCC) cases are associated with liver cirrhosis developing on a background of hepatic inflammation [1,2]. Moreover, it is generally accepted that upregulation of toll like receptor (TLR) signalling pathways accompany chronic liver disease progression[1]. TLRs play a critical role in innate immune responses[3] via activation of transcription factors such as nuclear factor (NF)-κB, activating protein-1 and interferon regulatory factors in inflammatory cell types, especially macrophages. For example, TLR-4 and TLR-9 play a pivotal role in non-alcoholic fatty liver disease (NAFLD) progression[4] and mediate neutrophil dysfunction in alcoholic hepatitis[5]. In addition, TLR-2 and TLR-9 contribute to alcohol-mediated liver injury through induction of CXCL1 and neutrophil infiltration[6]. Furthermore, activated stellate cells are involved in the development of liver fibrosis in carbon tetrachloride (CCl_4) treated mice through HMGB1-TLR2/4-NF-κB signalling pathways[7]. Recently, a role for TLR-2 in HCV progression has been described. It was found that a combination of TLR2 and IL28B polymorphisms not only had an influence on HCV viral load but was also responsible for HCV disease progression[8]. More recently, IL-37 was reported to exert its anti-inflammatory action through inhibition of MyD88-driven TLR-2 and TLR-4[9]. In addition to liver disease TLRs have also been implicated in cancer progression and chemoresistance [10]. We previously showed that TLR-7 and TLR-9 are upregulated in HCC and demonstrated a positive correlation with HCC proliferation[11]. However, the roles of TLR-2 in chronic liver disease progression and hepatocellular carcinoma development have not been thoroughly investigated. We therefore, decided to examine TLR-2 expression in different types of liver disease. including viral hepatitis, cirrhosis and HCC, and determine whether there

was any correlation between expression of TLR2 and other markers of malignancy in HCC

MATERIALS AND METHODS

Patients

Human liver tissue samples used in this study were from a custom-made human formalin-fixed paraffin-embedded (FFPE) liver tissue array (V Biolabs, UK). The sections were generated in a serial manner slide 1, 2, ect. The array included liver samples with viral hepatitis (n=19), cirrhosis (n=21) and HCC (n=41) as well as normal liver tissue (n=8). Normal liver in this setting has neither signs of inflammtion nor tumours. Haematoxylin and eosin staining was performed on the array in order to confirm the diagnosis of each sample.

Immunohistochemistry

Immuno-staining was performed on 4µm FFPE sections on the tissue microarray. The following primary antibodies from Abcam (UK) were used with overnight incubation at 4ºC: rabbit anti-TLR-2 polyclonal (1:100 dilution); rabbit anti-Caspase-3 polyclonal (1:100), rabbit anti-Ki-67 monoclonal (1:100), and rabbit anti-VEGF polyclonal (1:100). Secondary antibody (Dako EnVision detection kit peroxidase/DAB, Rabbit/mouse, Dako, UK) for Ki-67 and Caspase-3, or secondary antibody (Invitrogen, alkaline phosphatase, Human, Invitrogen, UK) for TLR-2 and VEGF were applied for 30 minutes at room temperature. Semi-quantitative evaluation (signal intensity and localisation) of staining was performed by two independent liver pathologists. The scoring system used for TLR-2, Caspase-3 and VEGF stained slides is as described

in Table 1. Cytoplasmic staining was scored - depending on staining intensity –as either weak (+), moderate (++) or strong (+++) (Table 1). Proliferation index was used for quantification of Ki-67 expression; positively stained nuclei among 1,000 hepatocytes were counted in the highest expression area using a standardised grid and estimated as percentage.

Cell proliferation assay

Proliferation of the human HCC line HUH7 was measured using CellTiter 96 Aqueous one solution Cell proliferation Assay (Promega, USA). About $0.3X10^5$ HUH7 cells were cultured in 96 well plates and incubated with a TLR-2 agonist (Heat Killed Listeria monocytogenes; Invivogen, UK) at a concentration of 10^8/ml for 48 h.

Western blot

Rabbit polyclonal primary antibodies were used; VEGF (1:200 dilution, Santa Cruz, Germany), CD34 (1:1000, Cell Signalling Technology, Germany), PCNA (1:200 dilution, Santa Cruz, Germany) and Mouse monoclonal GAPDH (1:1000 dilution, Santa Cruz, Germany). Peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were used (1:5000 dilution, Santa Cruz, Germany).

Hundred microliter of mammalian protein extraction reagent M-PER (Thermo Fisher Scientific, Germany) were added to HuH7 cells grown in 6 wells plates. Twenty micrograms of protein from each well were prepared and loaded onto Nupage gels (Thermo Fisher Scientific, Germany).

Caspase-3 assay

The activity of Caspase-3 enzyme was fluorometrically determined as previously described [12] with minor modifications. Twenty-five microliter of cell lysate, were added to 10 µl of AC-DEVD-AFC fluorogenicCaspase-3 substrate (Biomol, Hamburg, Germany) and made up to 100 µl with assay buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 µM EDTA, and 10% glycerol). Following incubation at 37°C in the dark for 60 min, fluorescence was measured with a Tecan infinite M200 microplate reader (Thermo Scientific, Waltham, MA, USA). Protein concentration was determined using the DC protein assay (Bio-Rad, Munich, Germany). The measured fluorescence values were normalized to the protein content in each sample.

Statistical analysis

Continuous variables were analysed with Student's t-test normal distribution. For inter-group comparison, Mann-Whitney-U-test or Kruskal-Wallis test were used for the non-parametric analyses. Spearman rank correlation was used.

Results

TLR-2 localisation along disease dynamics in human liver tissue

We first examined the subcellular localisation of TLR-2, specifically the nuclear and cytoplasmic signals, in healthy (n=8), viral hepatitis (n=19), cirrhosis (n=21), and HCC (n=41) livers using IHC. Gender, age and hepatitis viral infections (B and C) revealed no significant differences between groups in the various disease stages (Table 2, supp. 1). In healthy livers, TLR-2 was found exclusively localised in the cytoplasmic compartment though the signalling was weak (score +) (Table 3, Figure 1). This faint expression further decreased with liver disease progression, the calculated percentages are 52.6, 38.1 and 28.6 in viral hepatitis, cirrhosis and HCC, respectively (Table 3, Figure 1). In the contrary, regions with moderately stained cytoplasmic TLR-2 (score ++) are increasing with disease stage severity with percentages of 10.5, 14.3 and 30.9 in viral hepatitis, cirrhosis and HCC, respectively (Table 3, Figure 1). No alterations are obvious in case of strong cytosolicTLR-2 signals (score +++) between cirrhosis and HCC patients, the only groups displaying those. Expression of TLR-2 in the nuclear compartment occurs in 10.0%, 14.3% and 30.9% of hepatitis, cirrhosis and HCC samples, respectively (Table 3). Moreover, our results indicate thatTLR-2 localisation changes during disease progression with more pronounced nuclear presence in the progressed disease stages cirrhosis and HCC.

TLR-2 expression is positively correlated with cell proliferation, independent of its subcellular localisation

In order to determine whether there is any correlation between TLR2 subcellular distribution and cell proliferation, we immunostained the liver tissue arrays with antibodies directed against Ki-67. As expected, the percentage of cells with Ki-67

positive nuclei was higher in hepatitis, cirrhosis and HCC tissue compared with healthy liver. In addition, we found a significant correlation between Ki-67 positivity and cytoplasmic TLR-2 expression (r=0.24; P=0.02) (Figure 2A, B). In the case of nuclear TLR2 the correlation with Ki-67 expression was even more pronounced (r=0.27; P=0.007) (Figure 3A, B). The functional studies carried out on HUH7 cells using the TLR-2 agonist demonstrated a significant increase in proliferation of HUH7 cells compared to control, untreated cells upon activation of TLR-2 signalling. This was measured by proliferation assay (P=0.03) (Figure 3C) and PCNA expression by western blot (Supp 2). Our data reveal that TLR-2 expression is not randomly distributed but is positively correlated with cell division in human liver and HCC cell line.

CytoplasmicTLR-2 expression is correlated with VEGF positive staining

In order to ascertain whether there is any association between expression of TLR-2 and tumour vascularisation, we also examined the expression of VEGF in our tissue samples. Despite finding a heterogeneous distribution of VEGF positive signalling was detected in parenchymal and inflammatory cells (Figure 4), we managed to determine a positive correlation between VEGF staining and TLR-2 expression, both nuclear and cytoplasmic, (Figure 4A, B), whereby though the significance was higher with cytoplasmic expression of TLR2 than nuclear (r=0.60; P<0.001 vs r=0.23; P=0.03 respectively) (Figure 4A, B). These results were consistent with the *in vitro* data from HUH7 cells which demonstrated upregulation of CD34 and VEGF expression following stimulation with TLR2 agonist (Figure 4C, D and supp 3). These findings suggest that cytoplasmic and nuclear expression of TLR-2 may play a role in tumour vascularisation.

TLR-2 is positively associated with Caspase-3 expression independent of its subcellular localisation.

The human liver tissue arrays were stained for Caspase-3 and a blinded correlation analysis with TLR-2 immunostaining was performed. The number of caspase 3 positive samples was found to be higher in hepatitis (6/19), cirrhosis (7/21) and HCC tissues (24/41) compared with healthy liver samples (1/8). No significant difference in Caspase 3 expression was observed between hepatitis and cirrhotic liver tissue, both demonstrated Caspase-3 positivity in approximately one-third of cases. In HCC a significant positive correlation was observed between Caspase 3 expression and cytoplasmic TLR2 staining ($r=0.38$; $P<0.001$; Figure 5 A, B). A similar correlation between caspase3 and nuclear TLR-2 expression was also seen ($r=0.37$; $P<0.001$; Figure 5A, B). In other words, there is a significant association between Caspase 3 and TLR2 expression independent of its subcellular localisation.

However, when we looked at the Caspase-3 activity *invitro,* we did not observe a clear difference between HUH7 cells treated with TLR-2 agonist and control untreated cells (Figure 5 C). Our findings suggest that TLR-2 may increase the expression of Caspase-3 in HCC but this does translate into an increase in its activity.

Discussion

Toll-like receptors (TLRs) are pattern recognition receptors which induce pro-inflammatory signals and modulate the immune response[13,14]. However, persistent inflammation and immune response modulation create an appropriate microenvironment for tumour development suggesting that TLRs bridge the gap between inflammation and carcinogenesis [15]. In the context of the liver, inflammation based-disease usually progresses from mild to severe liver damage followed by fibrosis, cirrhosis and finally hepatocellular carcinoma (HCC). We have previously reported that expression of TLR-7 and TLR-9 positively correlates with proliferation of HCC cells *in vivo and in vitro* [11]. In this study, we demonstrated TLR-2 is expressed in both the cytoplasm and nucleus of liver samples with various disease. In addition we have shown there is a significant correlation between expression of TLR-2 and the expression of proliferation, apoptosis and vascularisation markers in HCC.

In liver tissue with hepatitis and cirrhosis we have shown that TLR-2 is significantly accumulated in the nuclear area and strong expression can also be detected in the cytosol, as had previously been reported[16]. In contrast to our findings, Soares and colleagues (2012) reported the expression of TLR-2 to be reduced in HCC compared to liver with hepatitis and cirrhosis. However, their study included a very small number of HCCs, only 6 in total. In addition, the background of references in their study was NAFLD.

Upregulation and nuclear translocation of TLR-2 are suggesting that TLR-2 plays a role in precancerous and carcinogenic stages as described in case of Toxoplasma gondiiinfection induced acute damage[17], acute fulminant viral hepatitis[18]and

chronic liver injury[19], ischemia/perfusion rat model[20] and alcohol-driven hepatitis[6,21].

TLR-2 expression has also been demonstrated to be elevated in intrahepatic cholangiocarcinoma. Moreover, this increased level of expression was also shown to correlate with tumour invasion [22].

These findings are similar to In line with our finding, a strong positive correlation was reported between cell proliferation index and the cytosolic expression and nuclear translocation of TLR-2 particularly in HCC patients.

In addition it has been shown that downregulation of TLR-2 using siRNA leads to a significant inhibition of cell proliferation[23] in HepG2 cells. Furthermore, miR-143 downregulates TLR-2 expression in hepatoma cells which in turn inhibits hepatoma cell proliferation[24]. The link between TLR-2 and cell proliferation has previously also been reported in embryonic stem cells by Taylor et al [25]. In vitro functional studies Another study in vitro model of non-small cell lung cancer reported that TLR-2 agonist (lipotechoic acid) and Listeria monocytogenes stimulate cell proliferation[26] and promote tumour growth[27] respectively . Furthermore, this positive correlation between TLR-2 expression and cell proliferation was confirmed in various organs e.g. mouse kidney[28] and pancreatic cancer cells[29]. These aforementioned results and our findings are consistent and suggesting that TLR-2 plays-so far not well studied- a role in cell proliferation. Interestingly, the expression of vascular endothelial growth factor (VEGF) was positively correlated with TLR-2 and this correlation was more significant with cytoplasmic than nuclear TLR-2 expression, suggesting its role when it is found in the cytoplasm to enhance angiogenesis. A previous study on Human umbilical vein endothelial cell (HUVEC) infected with Chlamydia pneumoniae reported that TLR-2 expression is positively correlated with the production of VEGF[30]. In

addition, the association between TLR-2 expression and angiogenesis promotion was observed in mesenchymal stem cell[31]. Furthermore, a significant positive association was recorded between upregulation of TLR-2 expression at the mRNA and protein levels and VEGF in human gastric carcinoma[32]. It has been shown also stimulation of TLR-2 resulted in upregulated VEGF gene expression in three human pancreatic cancer cell lines[29].

Current study shows a positive and significant correlation of TLR expression with apoptotic marker Caspase-3. Previously, it has been shown that TLR-2 is a novel apoptosis pathway modulator[33] through MyD88. Moreover, TLR-2 is described as a sensor for apoptosis in a mouse model of acute inflammation[34] and intrahepatic apoptosis was significantly diminished in $TLR2^{-/-}$ mice in bile duct ligation model[35]. Recently, it was reported that persistent hepatocyte apoptosis is a crucial factor for the development of HCC. In this study, authors reported that Caspase-8 achieves not only apoptosis but also maintains HCC proliferation through DNA damage associated response[36]. Moreover, TLR-2 as an apoptotic mediator was proved in microglia with herpes infection[37].

In a previous study, expression of Caspase-3 was not correlated with incidence of apoptosis but it was correlated with poor prognosis in resected non-small cell lung cancer NSCLC [38]. This can explain the increased Caspase-3 expression in our HCC cases.

In conclusion, the current study has some limitations, including the type of tissue sample and its number. Despite that, our data suggest that TLR-2 plays a critical role in liver disease progression, HCC development and maintenance might be via proliferation, and angiogenesis modulation. Further studies are required to identify the

possible cross- talks between TLR-2 expression/nuclear translocation and HCC development and progression.

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Figure legends

Figure 1: Localisation and intensity of TLR-2 signals during disease dynamics. Livers from control, hepatitis, cirrhosis and HCC are evaluated using antibody directed against TLR-2. Overviews and close-ups are presented using 10X and 40X, respectively.

Figure 2: Expression of TLR-2 is correlated with proliferation, apoptosis and vascularisation in HCC. Livers HCC patients are evaluated using antibody against TLR-2, Ki-67, Caspase-3 and VEGF as a marker for proliferation, apoptosis and vascularisation, respectively. Overviews and close-ups are presented using 10X and 40X, respectively.

Figure 3: Expression of TLR-2 is correlated with HCC proliferation. A) Livers from HCC are tested using antibody detecting TLR-2 and Ki-67. Significant positive correlations between cytoplasmic/nuclear TLR-2 and Ki-67 positive nuclei as indicated by correlation coefficient (r) are reported. Overviews and close-ups are presented using 10X and 40X, respectively. **B)** TLR-2 localisation is positively correlated with Ki-67. **C)** Using TLR-2 agonist, HUH7 proliferation is significantly increased. Number of experiments = 4 and 3 replicates for each condition.

Figure 4: Expression of TLR-2 is associated with vascularisation markers in HCC

A) Livers from HCC are tested using antibody directed against TLR-2, and VEGF. VEGF expressed in inflammatory cells (yellow arrows) and malignant hepatocytes. **B)** Significant positive correlations are found between cytoplasmic/nuclear TLR-2 and VEGF positivity as indicated by correlation coefficient (r). **C** and **D**) HUH7 cells are stimulated with TLR-2 agonist and CD34 and VEGF are investigated by western blotting.

Figure 5: Expression of TLR-2 is positively correlated with HCC apoptosis. A) Livers from HCC are stained using antibody directed against TLR-2 and Caspase-3. **B)** Significant positive correlations are reported between cytoplasmic/nuclear TLR-2 and Caspase-3 as indicated by correlation coefficient (r). **C)** There is no difference in apoptosis assay of HUH7 stimulated cells with TLR-2 agonist.

Supplementary Figures :

1) Nuclear and cytoplasmic expression of TLR-2 in stage 2 and stage 3 of HCC cases.

TLR2 expression was higher in T3N0M0 than T2M0N0 but does not the significant level.

2) TLR-2 stimulation is associated with increased PCNA expression in HuH-7 cells.

HuH-7 cells are treated with TLR2 agonist and PCNA are investigated by western blot.

3) Increase VEGF expression after TLR-2 agonist treatment.

HUH7 cells are stimulated with TLR-2 agonist and VEGF is investigated by Immunofluorescence