Myeloid Differentiation Factor 88 Promotes Growth and Metastasis of Human Hepatocellular Carcinoma

Beibei Liang1,2, Rui Chen2, Tao Wang2, Lei Cao1,2, Yingying Liu2, Fan Yin2, Minhui Zhu2, Xiaoyu Fan2, Yingchao Liang2, Lu Zhang2, Yajun Guo1,2,3, and Jian Zhao2,3

Abstract

Purpose: To investigate the expression of myeloid differentiation factor 88 (MyD88) in hepatocellular carcinoma (HCC) and its prognostic value in patients with HCC.

Experimental Design: Expression of MyD88 was detected by immunohistochemistry in surgical HCC specimens (n = 110). The correlation of MyD88 expression to clinicopathologic characteristics was analyzed. The involvement of MyD88 in tumor growth and invasion was investigated.

Results: The expression of MyD88 was significantly higher in HCC tumors than that in adjacent nontumor tissues. Particularly, high expression of MyD88 was found in HCCs with late tumor stage (P = 0.029). Patients with high MyD88 staining revealed a higher recurrence rate (65% vs. 40%; P = 0.008). Kaplan–Meier analysis showed that recurrence-free survival (RFS; P = 0.011) and overall survival (OS; P = 0.022) were significantly worse among patients with high MyD88 staining. Univariate and multivariate analyses revealed that MyD88 was an independent predictor for OS and RFS. Ectopic expression of MyD88 promoted HCC cell proliferation and invasion in vitro. Suppression of MyD88 expression with lentivirus encoding short hairpin RNA reduced tumor growth and invasion, as well as lung metastasis. Finally, silencing of MyD88 inhibited the activation of NF-κB and AKT in HCC cells, whereas forced expression of MyD88 was able to enhance the activation of NF-κB and p38 extracellular signal–regulated kinase without Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) signaling.

Conclusion: Elevated expression of MyD88 may promote tumor growth and metastasis via both TLR/IL-1R–dependent and –independent signaling and may serve as a biomarker for prognosis of patients with HCC. Clin Cancer Res; 19(11); 2905–16. ©2013 AACR.

Introduction

Invasion and metastasis are the leading causes of death in patients with cancer. Inflammation is considered to be the most important environmental factor contributing to tumor progression by promoting proliferation, apoptosis, invasion, and angiogenesis (1–3). The inflammatory response can be initiated by several types of pattern-recognition receptors (PRR), the Toll-like receptors (TLR) are the well-characterized PRR (4). The interleukin (IL)-1 receptors (IL-1R) share a common Toll/IL-1 receptor (TIR) motif in their cytoplasmic domain with TLRs. TIR domain-containing adaptor proteins are required to bridge the TLR/IL-1R receptors to the intracellular molecules and transmit cellular signaling. The first such adaptor molecule to be discovered is myeloid differentiation factor 88 (MyD88;refs. 5–7). By interaction with TIR domain of TIR, MyD88 recruits IL-1R–associated kinase (IRAK) and TNF receptor–associated factor-6 (TRAF6), leading to activation of NF-κB and mitogen-activated protein kinases (MAPK; ref. 8).

There is increasing evidence for MyD88 playing an important role in carcinogenesis. Mice lacking MyD88 formed fewer tumors than wild-type (WT) mice in diethylnitrosamine (DEN)-induced hepatocarcinogenesis or azoxymethane (AOM)-induced intestinal tumorigenesis (9, 10). In DEN-induced hepatocarcinogenesis, MyD88 was found critical for the production of IL-6 in Kupffer cells (9). The contribution of MyD88 to inflammation-associated tumorigenesis was further confirmed in 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol 13-acetate (TPA)–induced skin papilloma (11). In addition to inducing proinflammatory response, recent evidence showed that MyD88 may act intrinsically in epithelial cells to promote carcinogenesis by noninflammatory functions. In APCMin mice, MyD88-dependent signaling was found required for positive regulators of tumor progression.
**Translational Relevance**

Tumor metastasis is the leading cause for the death in patients with hepatocellular carcinoma (HCC) after curative resection. Therefore, it is urgent to reveal the mechanism underlying HCC metastasis. Here, we show that the expression of myeloid differentiation factor 88 (MyD88), an adaptor molecule for Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) signaling, is enhanced in HCCs. Patients with high MyD88 staining reveal a higher recurrence rate and poorer recurrence-free survival and overall survival. MyD88 may promote tumor metastasis through regulation of apoptosis and expression of inflammation factors and other tumor modifiers such as matrix metalloproteinase 7 and COX2, via both TLR/IL-1R-dependent and -independent signaling. Our results suggest that MyD88 may be a candidate prognostic factor for HCC and a valuable target for therapy.

derived from epithelial cells, such as matrix metalloproteinase 7 (MMP7) and COX-2 (10). In addition, in methylcholanthrene (MCA)-induced fibrosarcoma, a model that has not been classically defined as having a significant inflammatory origin, fewer MyD88−/− mice developed fibrosarcoma than WT control. MyD88 might act intrinsically to facilitate fibroblast and epithelial cell to transformation (11). The direct evidence of intrinsic MyD88 in the regulation of carcinogenesis by noninflammatory response came from a Ras-dependent skin carcinogenesis model (12). MyD88 was found to play a cell-autonomous role in the regulation of cell-cycle checkpoint and proliferation via its interaction with activated extracellular signal–regulated kinase (ERK). Thus, MyD88 may have multiple facets in tumorigenesis, via both proinflammatory and noninflammatory responses.

Abnormal expression of MyD88 has been previously reported in various types of cancer, which is related to tumor development and chemoresistance. High expression of MyD88 was found in stomach, colon, and lung primary human cancer tissues, as well as in papillomas developed from DMBA/TPA–treated mice (12). In colorectal cancer (CRC), high expression of MyD88 was frequently detected in CRC with liver metastasis and significantly related to poor prognosis of patients with cancer (13). Upregulation of MyD88 was also found in ovarian cancer cells and in ovarian cancer tissues (14, 15). SKOV3, a cell line obtained from the ascites of a patient with advanced, metastatic ovarian cancer, expresses high level of MyD88 (15). Patients with ovarian cancer whose tumors did not express MyD88 had a statistically significant improved progression-free interval compared with patients whose tumors expressed MyD88 (14). Moreover, high expression of MyD88 conferred ovarian cancer cells resistance to chemotherapy (14, 16). Patients whose tumor was MyD88-positive had a poor response to paclitaxel chemotherapy (16). Recently, RNA interference screening revealed that somatically acquired MyD88 mutations in activated B-cell–like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) activated NF-kB and JAK–STAT3 signaling to promote cell survival (17). The development of human hepatocellular carcinoma (HCC) is closely associated with chronic inflammation. Evidences have shown that TLR/IL-1R signaling plays an important role in hepatocarcinogenesis (18, 19). Recent evidence showed that TLR4 single-nucleotide polymorphisms might be associated with the development of HCC (20, 21). However, little is known about the expression of MyD88 in human HCCs and its correlation with tumor development. In this study, we analyze the expression of MyD88 in 110 cases of HCCs and evaluate its correlation with clinicopathologic characteristics. The effects of hepatic MyD88 on cell survival, proliferation, and invasion are assessed in vitro as well as in vivo.

**Materials and Methods**

**Patient samples**

In all, 110 primary HCC samples with adjacent nontumors liver tissues were obtained from patients who had undergone curative hepatic resection between 2003 and 2006 at Guangxi Cancer Hospital (Nanning, Guangxi, PR China). Patient’s consent and approval from Guangxi Cancer Hospital Ethics Committee were obtained to use these clinical materials for research purposes. The entering criteria of all patients were described as previously reported (22, 23). Curative resection was defined as complete resection of all tumor nodules and the cut surface being free of cancer by histologic examination (23, 24). The clinicopathologic characteristics of the patients are summarized in Supplementary Table S1.

Patient follow-up was completed on March 15, 2011. The median follow-up period was 41 months (range, 1–79 months). All patients were monitored postoperatively by serum α-fetoprotein (AFP), abdominal ultrasonography, and chest radiograph every 1 to 6 months depending on the postoperative time as previously described (25, 26). If recurrence was suspected, the patients were detected by computed tomography and/or MRI. Recurrences were confirmed on the basis of typical imaging appearances in computed tomography scans and/or MRI and an elevated AFP level. Most patients died from intrahepatic recurrence, distal metastasis, or complicated cirrhosis.

**Immunohistochemical staining**

The expression of MyD88 was analyzed with EnVision system (Changdao Biotech, Shanghai, China) in formalin-fixed, paraffin-embedded sections of primary tumors. Briefly, the slides were dewaxed, hydrated and washed, and the endogenous peroxidase activity was quenched. After microwave antigen retrieval, slides were blocked and then incubated with the antibody against MyD88 (T3223, Epitomics) overnight at 4°C. Subsequently, sections were rinsed and incubated with the working solution of horseradish peroxidase–labeled goat anti-rabbit for 60 minutes at 37°C. After
rinsing for 3 times, staining was visualized using the peroxide substrate solution diaminobenzidine. Counterstained by hematoxylin, the slides were dehydrated in graded alcohol and mounted. Negative controls were prepared in the absence of primary antibody.

Evaluation of immunostaining was independently conducted by 2 experienced pathologists. The expression of MyD88 was scored according to the signal intensity and distribution. Briefly, a mean percentage of high tumor cells was determined in at least 5 areas at \( \times 400 \) magnification and assigned to 1 of 5 following categories: 0, <5%; 1, 5%–25%; 2, 25%–50%; 3, 50%–75%; and 4, >75%. The intensity of immunostaining was scored as follows: 1, weak; 2, moderate; and 3, intense. For tumors that showed heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of high tumor cells and the staining intensity were multiplied to produce a weighted score for each case. Tissues with immunohistochemical scoring 2 or less were considered as low, 3 to 12 as high.

**Cells and plasmids, siRNA, lentivirus**

PLC/PRF/5, HepG2, and Hep3B were purchased from American Type Culture Collection (ATCC). MHCC97-L and HCC-LM3 were obtained from the Liver Cancer Institute, Zhong Shan Hospital, Fudan University (Shanghai, PR China). HL7702, Huh-7, and SMCC-7721 were obtained from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, PR China). All these cell lines were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% (v/v) FBS (Hyclone) at 37°C in a humidified incubator. Tissues with immunohistochemical scoring 2 or less were considered as low, 3 to 12 as high.

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**Luciferase reporter assays and electrophoretic mobility shift assay**

HCC (3 \( \times 10^4 \)) cells were plated in 48-well plates and transfected with full NF-\( \kappa B \)-driven luciferase construct together with the pRL-TK in triplicate by FuGENE HD Transfection Reagent (Roche). We harvested cells 48 hours after transfection and conducted the luciferase assays using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were calculated as fold induction compared with that in pGL3.0. All bar diagrams are shown as the mean ± SD.

The nuclear extracts from cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). Detection of DNA–protein binding by electrophoretic mobility shift assay (EMSA) was done using LightShift chemiluminescent EMSA kit (Pierce). Double-stranded gel shift probes corresponding to the human consensus NF-\( \kappa B \) sequences2 (5’-AGTGGGAGGACCTTCCAGGC-3’) were end-labeled with biotin.

**Plate colony formation, cell invasion, and migration assay**

Forty-eight hours after transfection, cells were dispersed into single-cell suspension, which was prepared and inoculated in 100-mm dishes with a density of 5 \( \times 10^4 \) cells and maintained for 12 days. Afterward, the colonies were stained with 1% crystal violet for 30 seconds after fixation with 4% paraformaldehyde for 5 minutes, and the colonies were counted. Each experiment was repeated in triplicate. The results presented are averages from 3 independent experiments.

Ability of cell invasion and migration was evaluated by the Cell Invasion Assay Kit (Millipore) and Transwell Permeable Support (Corning) according to the manufacturer’s directory. Five 200-multiple microscopic fields were randomly selected to calculate the total count of the invaded or migrated cells. The relative number of cells having penetrated the ECM or basement membrane was used to denote the invasion or migration ability of the cells. All assays were conducted 3 times.

**Detection of apoptosis**

Apoptotic cells were analyzed by the ApoDETECT Annexin V–FITC Kit (Invitrogen) in vitro. In situ apoptosis assay was conducted with the Fluorescein FragEL DNA Fragmentation Detection Kit (QIA39-1EA; Merck). The formalin-fixed paraffin sections were deparaaffinized and incubated with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture. Apoptotic cells carrying DNA labeled with fluorescein isothiocyanate (FITC)–dUTP were observed under fluorescence microscope (Olympus).
Animal studies

Male athymic BALB/c nude mice were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, PR China) and were maintained in specific pathogen-free conditions. Animal care and experimental protocols were conducted in accordance with the guidelines of Shanghai Medical Experimental Animal Care Commission. For in vivo treatment, HCC-LM3 cells (5 × 10⁶) infected with lentivirus encoding shMyD88 [at a multiplicity of infection (MOI) of 50] were implanted subcutaneously into the flank of nude mice (6 in each group, male BALB/c nu/nu, 4–6 weeks), and HCC-LM3 cells treated with lentivirus encoding shNon were used as mock control. Tumor growth was monitored with tumor volume, which was calculated as described before (27). The mice were sacrificed 6 weeks later, and the lungs were removed. Consecutive sections were made for every tissue block of the lung and stained with hematoxylin and eosin (H&E). The incidence and classification of lung metastasis were calculated and evaluated independently by 2 pathologists.

Statistical analysis

All statistical analyses were carried out using SPSS 16.0 for Windows software. The χ² test was used to compare qualitative variables; quantitative variables were analyzed by two-tailed Student t test and Wilcoxon rank sum test. Clinical variables included age, gender, hepatitis B virus (HBV) active status, AFP, cirrhosis, tumor number, vascular invasion, tumor size, and stage. Tumor stage was determined according to the American Joint Committee on Cancer (AJCC) classification system. Kaplan–Meier analysis was used to determine the survival data. Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last observation point. For surviving patients, the data were censored at the last follow-up. Recurrence-free survival (RFS) was defined as the interval between the date of surgery and the date of diagnosis of any type of relapse (intrahepatic recurrence and extrahepatic metastasis). Difference in survival between groups was evaluated by the log-rank test. Univariate and multivariate analyses were based on the Cox proportional hazards regression model. Data were presented as the mean ± SEM. All statistical tests were two-sided, and P < 0.05 was considered statistically significant.

Results

Enhanced expression of MyD88 is associated with poor prognosis in HCC patients

The expression of MyD88 was examined in 110 hepatocarcinomas and adjacent nontumor tissues using anti-human MyD88 antibody (Fig. 1A). Immunostaining of MyD88 was mainly detected in the cytoplasm of hepatic cells. Most of the stroma cells were negative staining, although sporadic positive staining on these cells was also observed (Fig. 1A and Supplementary Fig. S1A–S1C). High staining of MyD88 could be observed in 57 of 110 (51.8%) cases of HCCs, whereas MyD88 showed high staining in only 10 of 110 (9.1%) cases of adjacent nontumor tissues. Statistical analysis revealed that the immunostaining scores in tumor tissues [score value: median, 9 (range, 2–12)] were significantly higher than that in adjacent nontumor tissues [score value: median, 6 (range, 2–12); P < 0.001]. Considering the relationship between MyD88 expression levels and inflammation, CD68 was stained in MyD88-high and MyD88-low groups to detect the levels of inflammation. The average amounts of CD68⁺ cells in intratumor or in...
peritumor were similar with no significant difference between HCC tissues with high or low MyD88 expression (Supplementary Fig. S1).

Statistical analysis showed that MyD88 expression was not significantly correlated with age, gender, hepatitis B surface antigen (HbsAg), serum AFP level, cirrhosis, vascular invasion, and tumor number (Table 1). However, its expression level was found to be significantly higher in HCCs with AJCC stage III–IV than HCCs with stage I–II ($P = 0.029$). Notably, patients with high MyD88 staining revealed a higher recurrence rate after surgical resection (65% vs. 40%; $P = 0.008$; Table 1).

The potential association between MyD88 expression level and RFS or OS was retrospectively evaluated. Kaplan–Meier analysis showed that RFS ($P = 0.011$; Fig. 1B) and OS ($P = 0.022$; Fig. 1C) were significantly worse among patients with high MyD88 staining. Patients in MyD88-high group had less median cancer-free survival than patients in MyD88-low group (14 vs. 38 months). The OS estimates showed that patients in MyD88-high group had less median (32 vs. 42 months) and lower OS (37.5% vs. 66.7%) than patients in MyD88-low group. Consistently, the 1-, 3-, and 5-year OS and RFS after surgery were much worse for patients with MyD88-high than patients with MyD88-low. RFS and OS (in brackets) rates at 1, 3, and 5 years posthepatectomy were 74% (83%), 55% (66.5%), and 48% (54%) in MyD88-low expression group and were 55% (77%), 31.5% (43.8%), and 26% (23.9%) in MyD88-high expression group (Supplementary Table S2). In univariate analysis, vascular invasion and MyD88 expression status were prognostic factors for RFS and OS (Table 2). Multivariate analysis revealed that MyD88 expression status was defined as an independent prognostic for both RFS ($P = 0.027$) and OS ($P = 0.026$). MyD88-high expression patients were about 1.8 times more likely to suffer from relapse than MyD88-low expression patients (Table 2). Thus, increased expression of MyD88 may serve as a prognostic indicator for patients with HCC.

**Enhanced MyD88 promotes proliferation and survival of HCC cells**

The mRNA level of MyD88 was markedly higher in 5 HCC cells compared with HL7702, a normal hepatic cell (Fig. 2A). Moreover, the level of MyD88 in HL7702 was markedly elevated by the stimulation of lipopolysaccharide (LPS), IL-1α, or IL-1β (Supplementary Fig. S2A). We then used lentivirus-encoding shRNA to knockdown MyD88 in HepG2 with high level of MyD88 and HCC-LM3 with medium level of MyD88, or pcDNA3.0 vector encoding MyD88 cDNA to overexpression MyD88 in HepG2 with high level of MyD88 and HCC-LM3 with medium level of MyD88, or pcDNA3.0 vector encoding MyD88 cDNA to overexpression MyD88 in HepG2 and HCC-LM3 cells (Supplementary Fig. S2B and S2C). Plate colony formation assay showed that knockdown of MyD88 greatly inhibited cell proliferation in HCC-LM3 and HepG2 cells, whereas overexpression of MyD88 markedly enhanced cell proliferation in HL7702 and HCC-LM3 cells (Fig. 2B). The effect of MyD88 on the cell growth was further confirmed by in vitro assay in HCC-LM3 xenografts. As shown in Fig. 2C, depletion of MyD88 in HCC-LM3 cells dramatically inhibited tumor growth in nude mice. The tumor volumes developed by MyD88-deficient HCC-LM3 cells were about half of that developed by control cells.

Evading apoptosis has been regarded as one of the cellular mechanisms contributing to the development of cancer (28). We then investigate whether enhanced MyD88 expression in HCC leads to resistance to apoptotic stimulation. The percentage of apoptotic cell greatly increased with the overexpression of MyD88 in HCC-LM3 cells (Fig. 2D). Apoptosis was further examined by in situ TUNEL assay on HCC-LM3 xenografted tumor tissues. More apoptotic nuclei, seen as green color excited under fluorescence

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**Table 1.** The associations of MyD88 expression with clinicopathologic characteristics in 110 patients with HCC

<table>
<thead>
<tr>
<th>Feature</th>
<th>Low ($n = 53$)</th>
<th>High ($n = 57$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.305</td>
</tr>
<tr>
<td>Male</td>
<td>45</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td>0.488</td>
</tr>
<tr>
<td>&lt;50</td>
<td>31</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>22</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>HbsAg</td>
<td></td>
<td></td>
<td>0.227</td>
</tr>
<tr>
<td>Positive</td>
<td>47</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AFP, ng/mL</td>
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</tr>
<tr>
<td>≤400</td>
<td>26</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>&gt;400</td>
<td>27</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
<td></td>
<td>0.670</td>
</tr>
<tr>
<td>–</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>43</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Tumor size, cm</td>
<td></td>
<td></td>
<td>0.790</td>
</tr>
<tr>
<td>&lt;5</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>≥5</td>
<td>35</td>
<td>39</td>
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</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td>0.148</td>
</tr>
<tr>
<td>No</td>
<td>45</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Tumor number</td>
<td></td>
<td></td>
<td>0.495</td>
</tr>
<tr>
<td>Single</td>
<td>51</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Multiple</td>
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<td>AJCC stage</td>
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<td>I–II</td>
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<td>III–IV</td>
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<tr>
<td>Recurrence</td>
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<tr>
<td>–</td>
<td>32</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>21</td>
<td>37</td>
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</table>

NOTE: $P$ values are two-tailed and based on the Pearson $\chi^2$ test.
Enhanced MyD88 promotes activation of NF-κB, PI3K/AKT, and p38/ERK in HCC cells

To explore the role of MyD88 in both TLR-dependent and -independent pathway, several genes that relate to tumor progression including MMP7, COX2, osteopontin (OPN), and proinflammatory cytokines such as IL-6, IL-1β, and TNF-α were analyzed with the presence or absence of IL-1β.

As expected, silence of MyD88 dramatically inhibited IL-1β-induced expression of these tumor modifiers in HCC-LM3 cells (Supplementary Fig. S3A and S3B). Interestingly, knockdown of MyD88 alone was sufficient to inhibit endogenous expression of MMP7, IL-1β, IL-6, and TNF-α. Moreover, forced expression of MyD88 was able to induce expression of these tumor modifiers to the level comparable with that induced by IL-1β (Supplementary Fig. S3C and S3D). The alterations of IL-1β, IL-6, and TNF-α were confirmed by ELISA assay (Supplementary Fig. S3E).

NF-κB is one of the main downstream signaling components of TLR/MyD88 signaling (8) and is constitutively activated in HCC (30). We then investigate whether enhanced expression of MyD88 leads to activation of NF-κB in HCC cells. As expected, knockdown of MyD88 greatly attenuated IL-1β-induced NF-κB activity in HCC-LM3, HepG2, and HCC-LM3 (Fig. 4A). Notably, depletion of MyD88 was able to significantly inhibit intrinsic NF-κB activity in MyD88-high expression cells HCC-LM3 and HepG2, whereas overexpression of MyD88 alone was able to enhance NF-κB transcriptional activity in these cells (Fig. 4A). The requirement of MyD88 for NF-κB activation in TLR/IL-1R–independent manner was further confirmed by DNA-binding activity analysis (Fig. 4B). Silencing of MyD88 attenuated NF-κB DNA-binding activity in MyD88-high HepG2 cells (Fig. 4B, left), whereas overexpression of MyD88 enhanced NF-κB DNA-binding activity in HCC-LM3 cells (Fig. 4B, right). Moreover, depletion of MyD88 inhibited phosphorylation of IkBα and its degradation independent of IL-1β stimulation, whereas forced expression of MyD88 enhanced phosphorylation of IkBα and its degradation in HCC-LM3 cells (Fig. 4C). These data suggest that enhanced expression of MyD88 is able to activate NF-κB via TLR/IL-1R–independent signaling in HCC cells.

Table 2. Univariate and multivariate analyses of factors associated with disease-free survival and OS

<table>
<thead>
<tr>
<th>Variables</th>
<th>RFS HR (95% CI)</th>
<th>P value</th>
<th>OS HR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate analyses</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gender (female vs. male)</td>
<td>3.151 (0.985–10.08)</td>
<td>0.053</td>
<td>1.036 (0.442–2.428)</td>
<td>0.936</td>
</tr>
<tr>
<td>Age, y (&lt;50 vs. ≥50)</td>
<td>0.791 (0.460–1.359)</td>
<td>0.396</td>
<td>0.589 (0.322–1.075)</td>
<td>0.085</td>
</tr>
<tr>
<td>HbsAg (negative vs. positive)</td>
<td>1.184 (0.428–3.275)</td>
<td>0.745</td>
<td>2.790 (0.677–11.492)</td>
<td>0.155</td>
</tr>
<tr>
<td>AFP, ng/mL (≤400 vs. &gt;400)</td>
<td>1.144 (0.673–1.944)</td>
<td>0.620</td>
<td>1.020 (0.588–1.770)</td>
<td>0.943</td>
</tr>
<tr>
<td>Cirrhosis (no vs. yes)</td>
<td>2.149 (0.922–5.013)</td>
<td>0.077</td>
<td>1.186 (0.559–2.518)</td>
<td>0.657</td>
</tr>
<tr>
<td>Tumor size, cm (&lt;5 vs. ≥5)</td>
<td>1.191 (0.677–2.097)</td>
<td>0.544</td>
<td>1.106 (0.615–1.989)</td>
<td>0.737</td>
</tr>
<tr>
<td>Tumor number (single vs. multiple)</td>
<td>1.1380(1.412–3.143)</td>
<td>0.804</td>
<td>0.797 (0.247–2.571)</td>
<td>0.704</td>
</tr>
<tr>
<td>AJCC stage (I–II vs. III–IV)</td>
<td>0.580 (0.335–1.004)</td>
<td>0.052</td>
<td>0.619 (0.351–1.094)</td>
<td>0.099</td>
</tr>
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<td>Vascular invasion (no vs. yes)</td>
<td>2.243 (1.277–3.939)</td>
<td>0.005</td>
<td>1.855 (1.040–3.306)</td>
<td>0.036</td>
</tr>
<tr>
<td>MyD88 (low vs. high)</td>
<td>1.959 (1.145–3.353)</td>
<td>0.014</td>
<td>1.913 (1.082–3.380)</td>
<td>0.026</td>
</tr>
<tr>
<td>Multivariate analyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular invasion (no vs. yes)</td>
<td>2.084 (1.184–3.669)</td>
<td>0.011</td>
<td></td>
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<tr>
<td>MyD88 (low vs. high)</td>
<td>1.841 (1.072–3.161)</td>
<td>0.027</td>
<td>1.913 (1.082–3.380)</td>
<td>0.026</td>
</tr>
</tbody>
</table>

NOTE: Multivariate analysis, Cox proportional hazards regression model. Variables were adopted for their prognostic significance by univariate analysis and no obvious correlation between each other.

microscope, were detected in MyD88-deficient HCC-LM3 xenografts than that in control xenografts (Fig. 2E). In consistence with in vitro analysis, more apoptotic cells could be observed in MyD88-low HCC tissues than that in MyD88-high tissues (Supplementary Fig. S2D). Thus, MyD88 may contribute to tumor progression through regulation of apoptosis.

Enhanced MyD88 promotes invasion and metastasis in HCC cells

We further investigated the role of MyD88 in tumor metastasis, which has been implicated from clinical data. HepG2 and HCC-LM3 cells migrated slower and had less ability to invade through Matrigel when MyD88 was knocked down (Fig. 3A and B). In contrast, HL7702 and HCC-LM3 cells migrated faster and had more invasive ability when MyD88 was overexpressed (Fig. 3C and D). HCC-LM3 cell has a high degree of pulmonary metastasis after subcutaneous injection (29), we therefore examined the effects of MyD88 on tumor metastasis in HCC-LM3 xenografts in vivo. Six weeks after transplantation, none of the mice developed lung metastasis when MyD88 was silenced. In contrast, 6 of 7 control mice developed lung metastasis (Fig. 3E and Supplementary Table S3). These data suggest that enhanced MyD88 may promote the motile and invasive abilities of HCC cells.

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In vitro analysis, more apoptotic cells could be observed in MyD88-low HCC tissues than that in MyD88-high tissues (Supplementary Fig. S2D). Thus, MyD88 may contribute to tumor progression through regulation of apoptosis.

Enhanced MyD88 promotes activation of NF-κB, PI3K/AKT, and p38/ERK in HCC cells

As expected, silence of MyD88 dramatically inhibited IL-1β-induced expression of these tumor modifiers in HCC-LM3 cells (Supplementary Fig. S3A and S3B). Interestingly, knockdown of MyD88 alone was sufficient to inhibit endogenous expression of MMP7, IL-1β, IL-6, and TNF-α. Moreover, forced expression of MyD88 was able to induce expression of these tumor modifiers to the level comparable with that induced by IL-1β (Supplementary Fig. S3C and S3D). The alterations of IL-1β, IL-6, and TNF-α were confirmed by ELISA assay (Supplementary Fig. S3E).

NF-κB is one of the main downstream signaling components of TLR/MyD88 signaling (8) and is constitutively activated in HCC (30). We then investigate whether enhanced expression of MyD88 leads to activation of NF-κB in HCC cells. As expected, knockdown of MyD88 greatly attenuated IL-1β-induced NF-κB activity in HCC-LM3, HepG2, and HCC-LM3 (Fig. 4A). Notably, depletion of MyD88 was able to significantly inhibit intrinsic NF-κB activity in MyD88-high expression cells HCC-LM3 and HepG2, whereas overexpression of MyD88 alone was able to enhance NF-κB transcriptional activity in these cells (Fig. 4A). The requirement of MyD88 for NF-κB activation in TLR/IL-1R–independent manner was further confirmed by DNA-binding activity analysis (Fig. 4B). Silencing of MyD88 attenuated NF-κB DNA-binding activity in MyD88-high HepG2 cells (Fig. 4B, left), whereas overexpression of MyD88 enhanced NF-κB DNA-binding activity in HCC-LM3 cells (Fig. 4B, right). Moreover, depletion of MyD88 inhibited phosphorylation of IκBα and its degradation independent of IL-1β stimulation, whereas forced expression of MyD88 enhanced phosphorylation of IκBα and its degradation in HCC-LM3 cells (Fig. 4C). These data suggest that enhanced expression of MyD88 is able to activate NF-κB via TLR/IL-1R–independent signaling in HCC cells.
Phosphoinositide 3-kinase (PI3K) and AKT has been implicated in TLR/IL-1R signaling pathway, and may regulate NF-κB activation in both TLR/IL-1R–dependent and –independent signaling (31–33). Here, we found that downregulation of MyD88 greatly inhibited the intrinsic activation of AKT in HCC-LM3 cells independent of IL-1β stimulation, overexpression of MyD88 or stimulation with IL-1β did not cause further activation of AKT in HCC-LM3 cells (Fig. 4D and E). Treatment of HCC-LM3 cells with PI3K inhibitor LY294002 significantly inhibited MyD88-induced NF-κB activation, indicating that MyD88-induced NF-κB activation is, at least in part, via PI3K/AKT (Supplementary Fig. S3F).

TLR/IL-1R–MyD88 signaling also leads to activation of MAPks such as c-jun-NH2-kinase (JNK), p38, and ERK (8). Here, we found downregulation of MyD88 inhibited IL-1β–induced p38 phosphorylation, whereas overexpression of MyD88 alone was able to enhance p38 phosphorylation to the level similar to the one stimulated by IL-1β in HCC-LM3 cells (Fig. 4D and E). It has been reported that MyD88 binds...
to ERK and prevents its inactivation by its phosphatase, MKP3, thereby amplifying the activation of the canonical RAS pathway in a TLR/IL-1R–independent manner (12). Indeed, here we found overexpression of MyD88 was able to enhance ERK activation without IL-1β stimulation (Fig. 4D and E). These results suggest that enhanced MyD88 may lead to activation of p38/ERK via TLR/IL-1R–independent signaling. Treatment of HCC-LM3 cells with p38 inhibitor SB2035 and ERK inhibitor U0126 greatly inhibited MyD88-induced NF-κB activation (Supplementary Fig. S3F). Moreover, U0126 inhibited the migration in MyD88-high expression cells HepG2 and HCC-LM3, and HCC-LM3 overexpressing MyD88 (Supplementary Fig. S4A and S4B). These data indicate that MyD88-induced NF-κB activation and invasion also involves ERK activation.

Taken together, these findings suggest that MyD88 may promote tumor progression through TLR/IL-1R signaling independent activation of NF-κB via PI3K/AKT and p38/ERK.

Discussion

The development of HCC is closely associated with chronic inflammation caused by viral infection, alcohol consumption, or hepatic metabolic disorders. Evidences
have suggested that TLR/IL-1R signaling plays an important role in various live disease and HCC (34). Recent evidences have indicated the involvement of MyD88 in the development of HCC. InDEN-induced inflammation-associated liver cancer model, loss of TLR4 or MyD88 decreased DEN-induced tumor development including the incidence, size,

Figure 4. MyD88 activates NF-κB, PI3K/Akt, and p38/ERK in HCC cells. A, ectopic expression of endogenous MyD88 in HL7702, HepG2, and HCC-LM3 increases NF-κB transcriptional activities with and without IL-1β (10 ng/mL) stimulation for 24 hours (top), whereas downregulation of MyD88 decreases NF-κB transcriptional activities (bottom). NF-κB activities are analyzed by the luciferase activities 48 hours after transfection. Data are indicated as the number of folds (n-fold) over that of pGL3.0, and shown as the mean±SD (, P < 0.05; **, P < 0.01). B, downregulation of endogenous MyD88 in HepG2 decreases NF-κB DNA-binding activities (left), whereas ectopic expression of MyD88 in HCC-LM3 increases NF-κB DNA-binding activities (right) as analyzed by EMSA. NS, nonspecific signal. C-E, Western blot analysis of the expression of p-IκB-α, total IκB-α, MyD88, p-Akt, total Akt, p-p38, total p38, p-Erk1/2, total Erk1/2, p-JNK, and total JNK in HCC-LM3 infected with shNon or shMyD88, and transfected with pcDNA3.0 or pMyD88, in the presence or absence of IL-1β (10 ng/mL). The cells were collected 24 hours after treatment and then analyzed by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for analysis in C-E.
and number of tumor (9, 18). Recently, HBx protein was found to stimulate MyD88 expression in hepatocytes to stimulate IL-6 expression (35). Collectively, these data indicate a strong contribution of TLR/MyD88 signaling to hepatocarcinogenesis. In current study, we showed that MyD88 was frequently upregulated in HCCs, which was closely related with the worse stage of tumor and the higher recurrent rate in patients with HCC. Moreover, the survival analysis revealed that patients with high expression of MyD88 had worse DFS and OS. Univariate and multivariate analyses revealed that MyD88 was a significant predictor for OS and DFS. In addition, in an external cohort’s data available in public database [National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus accession # GSE14520], the expression of MyD88 is significantly higher in 10 cases of HCC with portal vein tumor thrombi (PT) than that in metastasis-free HCCs (PN) at the time of surgery (PN/PT is 0.5731; *P* = 0.0009693; ref. 36). Our findings and previous observations strongly implicate that elevated MyD88 is involved in the tumor progression and may serve as a prognostic factor for patients with HCC.

Although Kupffer cells are considered the primary cells in liver to respond to TLR/IL-1R signaling, recent studies provide evidence of TLR/IL-1R signaling in hepatic nonimmune cell populations, including hepatocytes, biliary epithelial cells, endothelial cells, and hepatic stellate cells (34). Even though hepatocytes express very low levels of TLR2, TLR3, TLR4, and TLR5 and their responses are fairly weak in vivo (37), recent evidence suggests that TLR/MyD88 signaling is involved in the biologic or pathologic processes in hepatocytes. HBx stimulated IL-6 expression in hepatocytes via a MyD88-dependent manner (35). LPS suppressed glucose production in hepatocytes through the TLR4/MyD88/NF-κB pathway (38). In Plasmodium-infected hepatocytes, MyD88 was required for NF-κB activation and inducible nitric oxide synthase expression (39). Here, we showed that elevated hepatic MyD88 was involved in the growth and metastasis of HCC cells.

Elevated MyD88 expression has been found in parenchymal cells in various types of cancer (13–15, 35, 40), even though the mechanisms for MyD88 upregulation are largely unknown. Here, we found that the expression of MyD88 could be upregulated by the stimulation of LPS, IL-1α, and IL-1β. The contribution of MyD88 to tumor progression is mainly linked to its antiapoptosis ability (14, 40). Here, we have shown that besides the ability to evade apoptotic stimulation, elevated expression of MyD88 conferred HCC cells with enhanced abilities of migration and invasion. Furthermore, several metastasis-related genes such as MMP7, COX2, OPN, and inflammation factors such as IL-6, IL-1β, and TNF-α were regulated in a MyD88-dependent manner. Anti-IL-6 antibody was able to attenuate MyD88-induced enhanced migration ability in HCC-LM3 cells (Supplementary Fig. S4C and S4D). Our results reveal the importance of hepatic MyD88 in the regulation of tumor microenvironment and tumor progression.

Notably, the effects of MyD88 on tumor progression may be via both TLR/IL-1R-dependent and -independent signaling. On one hand, MyD88 was upregulated by the stimulation of LPS and IL-1 and was required for IL-1β-induced upregulation of tumor modifiers such as MMP7, COX2, IL-6, and TNF-α, and the activation of NF-κB and p38. On the other hand, overexpression of MyD88 alone was sufficient to regulate the ability to evade apoptotic stimulation, migrate and invade through matrix, the expression of tumor modifiers, and the activation of NF-κB and p38/ERK in HCC cells. Although HCC cells expressed low level of TLRs as detected by PCR, most of HCC cells expressed high level of IL-1R compared with hepatic cell line HIL7702 (Supplementary Fig. S5A and S5B). In the light of this, IL-1 is a major factor in the tumor microenvironment, thus enhanced MyD88 may promote tumor metastasis via IL-1R/MyD88 signaling in vivo.

However, the role of MyD88 in TLR/IL-1R–independent signaling during tumor development still needs to be elucidated. Previous studies have suggested that MyD88 may be involved in TLR/IL-1R–independent signaling. The upregulation of MMP7 in dysplastic epithelium was perturbed by MyD88 deficiency rather than by TLR2/TLR4 double deficiency in APC<sup>Min</sup> mice (9, 10). In RAS-mediated tumor development, MyD88 mutant that cannot interact with ERK, but not mutant that cannot interact with IRAK, lost the ability to regulate cell transformation, indicating the role of MyD88 in TLR/IL-1R–independent signaling (12). Interaction of MyD88 with p-ERK has been suggested to cause ERK activation and make contribution to RAS-mediated cell transformation. We assessed the potential link between MyD88 mRNA expression and RAS pathway activation by testing RAS target gene signatures available in Molecular Signature Database. In MyD88 gene set, there is a “BILD_HRAS_ONCOCGENIC_SIGNATURE,” which indicates that expression of MyD88 may be increased in the activation status of RAS oncogenic pathways (41). Here, we found ERK was phosphorylated when MyD88 was overexpressed in HCC-LM3 cells (Fig. 4E), however, knockdown MyD88 did not attenuate ERK activation (Fig. 4D). When we examined the RAS–MEK–ERK activation in HCC cells, we could not find a correlation between MyD88 expression and RAS pathway activation in HCC cells we tested (Supplementary Fig. S5C). Therefore, it would be interesting to extend this analysis in more HCC tissue samples. Nevertheless, ERK inhibitor U0126 inhibited cell migration in MyD88-high HCC cells HepG2 and HCC-LM3, as well as in HCC-LM3 overexpressing MyD88 (Supplementary Fig. S4A and 4B). These indicate that ERK activation contributes to MyD88-mediated HCC development via TLR/IL-1R–independent signaling.

Our data suggested that MyD88 may regulate TLR/IL-1R–independent signaling through activation of PI3K/AKT/NF-κB in HCC. Here, we found depletion of MyD88 greatly attenuated NF-κB transcriptional activity, DNA-binding ability, and AKT phosphorylation in HCC cells. PI3K/AKT signaling has been suggested as one of the regulators for NF-κB activation induced by TNF, IL-1, and LPS (31–33). AKT has been shown to induce activation of NF-κB through inhibitor of κB kinase α (IKKα) phosphorylation and...
liberation of IκBα or p65 phosphorylation independent of IκBα degradation [31, 32, 42, 43]. MyD88 has a binding motif YXXM for p85, a regulatory unit of PI3K. MyD88 has been shown to be associated with p85 by immunoprecipitation in LPS stimulated RAW 264.7 cells [44]. LTα-and Staphylococcus aureus–induced cPLA and COX-2 expression are mediated through the formation of a TLR2/MyD88/PI3K/Rac1 complex in human tracheal smooth muscle cells [45]. The association of MyD88 with p85 is also observed in the context of TLR5 signaling in intestinal epithelial cells and is required for TLR5-induced phosphorylation of Akt, NF-κB activation, and IL-8 production [46, 47]. However, whether MyD88 and p85 interaction participates in TLR/IL-1R–independent signaling during HCC progression still needs further investigation.

Here, we show that enhanced MyD88 expression is a strong indicator for more aggressive tumors and poorer clinical outcome in HCC. Elevated MyD88 may promote HCC metastasis through activation of NF-κB, PI3K/akt, and p38/ERK, regulation of apoptosis and tumor microenvironment through both TLR/IL-1R–dependent and -independent signaling. Moreover, high expression of MyD88 may serve as a prognostic factor and a therapeutic target for HCC.

References


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