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The Role of TLRs and their Polymorphisms in Human Diseases

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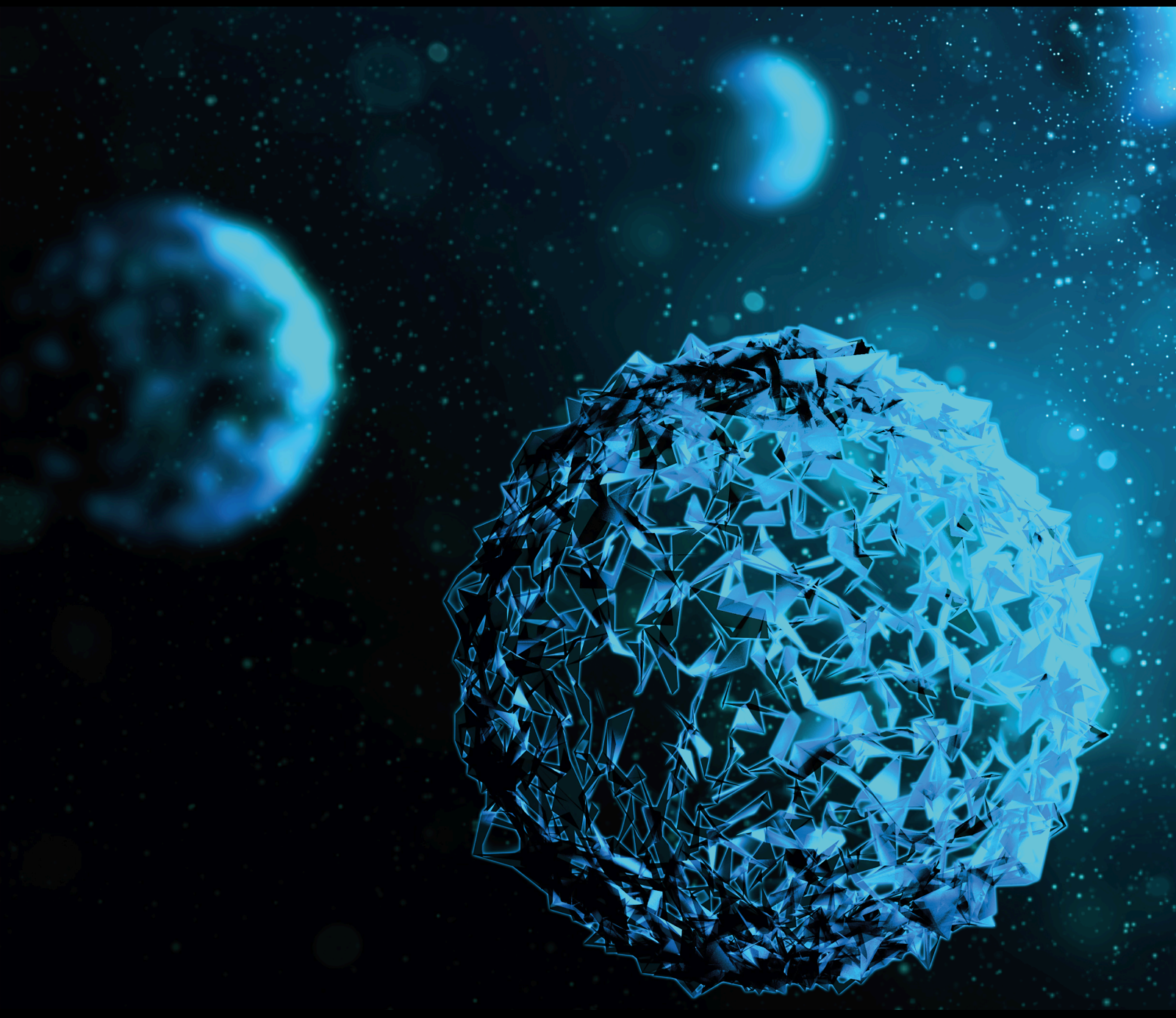

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Research Article

TAK-242 Ameliorates Hepatic Fibrosis by Regulating the Liver-Gut Axis

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Objective. The aims of this study were to investigate the impact of TAK-242 on the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear transcription factor-κB (NF-κB) signal transduction pathway in rats with hepatic fibrosis (HF) using the liver gut axis and to investigate the molecular mechanism of its intervention on HF. *Methods*. SPF grade SD male rats were randomly allocated to the control, model, and TAK-242 groups. For 8 weeks, the model and TAK-242 groups received 3 mL·kg^{-1} (the initial dose 5 mL·kg^{-1}) intraperitoneal injections of 40% CCL_4 olive oil solution. TAK-242 (5 mg·kg^{-1}) was administered once a day for 5 days after modeling. The pathological alterations of liver and small intestine tissues in each group were observed using H&E and Masson staining. ELISA was used to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL), total bilirubin (TBIL), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). RT-qPCR was utilized to identify the mRNA expression level of IL-1β, IL-6, TNF-α, TLR4, MyD88, and NF-κB in rat liver and small intestine tissues. The protein level of IL-1β, IL-6, TNF-α, TLR4, MyD88, and NF-κB protein in rat liver and small intestine tissues was determined utilizing Western blot and IHC. *Results*. TAK-242 significantly reduced AST, ALT, TBIL, and DBIL expression in HF rats' serum ($P < 0.01$) and alleviated liver tissue injury. Hematoxylin-eosin (H&E) and Masson staining revealed inflammatory cell infiltration and fibrous proliferation in the liver and small intestine tissue in the model group and partial cell swelling in the TAK-242 group, which indicated a considerable improvement compared to the model group. RT-qPCR, Western blot, and IHC data indicated that TAK-242 reduced the IL-1β, IL-6, TNF-α, TLR4, MyD88, and NF-κB expression in the liver and small intestine tissues of HF rats. *Conclusion*. TAK-242 might downregulate the TLR4/MyD88/NF-κB signal pathway through the liver-gut axis, suppress the inflammatory response, and eventually alleviate HF in rats.

1. Introduction

Hepatic fibrosis (HF) is a wound healing process induced by chemical toxic damage, chronic hepatitis virus infection, autoimmune liver disease, alcoholism, and other variables that result in aberrant production and deposition of liver extracellular matrix. [1] Clinically, even once the cause is eliminated, HF persists and may progress to cirrhosis, hepatocellular cancer, and, eventually, liver failure leading to death [2]. In contrast to irreversible cirrhosis, a growing number of investigations have shown that HF is a dynamical and probably bilateral process with an intrinsic possibility for recovery and remodeling [3, 4], providing many new ideas for anti-HF mechanism research and clinical treatment. Multiple studies have revealed that the liver-gut axis is usually linked to the advancement of liver disease [5, 6]. The intestinal mucosal barrier function is weakened when intestinal homeostasis is disrupted, resulting in a large influx of intestinal endotoxins into the liver via the portal system [7]. These bacterial products aggravate the development of fibrotic lesions in liver tissue by stimulating natural immune receptors, such as Toll-like receptors (TLRs).

It activates myeloid differentiation factor 88 (MyD88) to release serine-threonine protein 1 kinase (IRAK 1 kinase)

[8], which ultimately leads to the entry of nuclear transcription factor-κB (NF-κB) into the nucleus and ultimately activates downstream pathways involved in liver inflammation and fibrogenesis [5, 9]. It induces apoptosis in hepatic macrophages and the production of inflammatory molecules such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) [10–12], which exacerbate and destroy the intestinal barrier.

TAK-242 (Figure 1) is a small molecule of a toll-like receptor 4 (TLR4) that suppresses TLR4 activation by interacting directly with the intracellular domain of TIR [13]. Furthermore, TAK-242 shows hepatoprotective effects on Lipopolysaccharide/D-galactose (LPS/D-GalN)-induced fulminant hepatitis in mice [14], as well as suppression of TLR4 signaling to alleviate acute and chronic acute liver failure in animals. The TAK-242 has been demonstrated to minimize target organ damage and systemic inflammation in animal models [11], as well as ischemia/reperfusion injury in transplanted livers [15]; however, it is unclear if TAK-242 can specifically protect rats from HF through the liver-gut axis effects. This study evaluated the effect of TAK-242, a potential anti-inflammatory drug, in a CCl_4-induced HF rat model, revealed its mechanism of action, and identified a potential therapeutic target for clinical HF therapy.

2. Materials and Methods

2.1. Animals and Experimental Protocol. SD male rats (SPF grade), weighing 230 ± 10 g (10 weeks old), were supplied by Heilongjiang University of Traditional Chinese Medicine's Experimental Animal Center (animal certificate number: SYXK (black) 2018-007). The rats were kept in the following conditions: room temperature of 22 ± 2°C, relative humidity of 40%-60%, good ventilation, alternating light and dark light for 12 hours, standard feed, and free drinking water, which were provided to the experimental animals, and the Heilongjiang University of Traditional Chinese Medicine Ethics Committee approved the experiment (approval number DXLL2020081601). The rats were randomly divided into three groups of eight rats each after one week of adaptive feeding: control, model, and TAK-242 (MedChemExpress, HYB0000050025). The model and TAK-242 groups received intraperitoneal injections of 40% CCL_4 olive oil at 3 mL·kg^{-1} (5 mL·kg^{-1} for the first dose) [16, 17], while the control group received the same amount of olive oil twice a week for 8 weeks. After modeling, TAK-242 was administered once a day for 5 days with 10 %DMSO + 90% (20% SBE-β-CD in saline) at a dosage of 5 mg·kg^{-1} [14, 18–20]. The control and model groups received an equal amount of normal saline by gavage.

2.2. Sample Preparation. The rats were anesthetized with a 3% pentobarbital sodium solution. Blood was taken from the abdominal aorta and centrifuged for 15 minutes at 3500 r/min. The serum was isolated and refrigerated at -80°C for analysis. The liver and small intestine tissues were separated, the left two lobes of the liver and a part of the small intestine tissue were preserved with 4% paraformaldehyde solution, and the remainder were placed in a cryopreservation tube for later use.

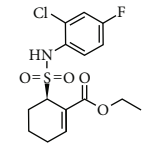

Figure 1: Chemical structure of TAK-242.

2.3. ELISA Detection Kits. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL), total bilirubin (TBIL), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were measured using an ELISA kit according to Nanjing Jiancheng Institute of Biological Engineering kit instructions and then analyzed with a microplate reader (Thermo Company).

2.4. Histopathological Staining

2.4.1. Hematoxylin-Eosin (H&E) Staining. Liver and small intestine tissues were fixed in 4% paraformaldehyde for more than 24 hours before being embedded using an alcohol gradient dehydration method. The embedded wax blocks were quickly sliced into sections 4 μm thick. Finally, the slices were stained with hematoxylin and eosin (H&E), and the pathological changes were examined using an optical microscope (Nikon Eclipse E100).

2.4.2. Masson Staining. For more than 24 hours, the liver tissue was immersed in 4% paraformaldehyde. The implanted wax blocks were quickly sliced into 4 μm thick slices and dewaxed. The slices were then immersed in Masson solution and sealed with neutral gum. A microscope (Nikon Eclipse E100, Japan) was used to examine the tissue collagen fiber area.

2.5. Real-Time qPCR. Total RNA was extracted from liver and small intestine tissues utilizing the trizol technique and dissolved in enzyme-free water, according to the experimental protocol. The total RNA was then reverse transcribed into cDNA using a reverse transcription kit, the PCR reaction system was prepared using ROX Reference Dye II, and quantitative real-time PCR was performed using the MyiQ™ Optics Module monochrome real-time PCR detection system (BioRad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the endogenous control. The relative quantification method was used to analyze the data, and the $2^{-\triangle\triangle Ct}$ method was used to analyze the data and determine the relative expression of mRNA. Table 1 displays a list of sequencing primers.

2.6. Immunohistochemical (IHC) Staining. The immunohistochemistry detection was carried out in exact compliance with the immunohistochemical kit's instructions. Tissues from the liver and small intestine were embedded and sectioned, then dewaxed, hydrated, and cleaned. The sections were then blocked with 3 percent H2O2 and goat serum

Table 1: Real-time qPCR sequencing primers.

Gene	Forward	Reverse	Reference
GAPDH	TTTGAGGGTGCAGCGAACTT	ACAGCAACAGGGTGGTGGAC	[21]
NF-κB	TGACGGGAGGGGAAGAAATC	TGAACAAACACGGAAGCTGG	[22]
TLR4	CCGCTCTGGCATCATCTTCA	CCCACTCGAGGTAGGTGTTTCTG	[23]
MyD88	TATACCAACCCTTGCACCAAGTC	TCAGGCTCCAAGTCAGCTCATC	[24]
TNF-α	CGTCGTAGCAAACCACCAAG	TTGAAGAGAACCTGGGAGTAGACA	[25]
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	[26]
IL-1β	TCGTGCTGTCGGACCCATAT	GGTTCTCCTTGTACAAAGCTCATG	[27]

after being treated with a pH 6.0 sodium citrate buffer solution. Then, add TLR4 (ab22048, Abcam), MyD88 (ab133739, Abcam), NF-κB (ab32536, Abcam), IL-6 (ab208113, Abcam), IL-1β (66737-1-Ig, Proteintech), TNF-α (ab1793, Abcam), ZO-1 (66452-1-Ig, Proteintech), and Claudin-1 (ab211737, Abcam) were stained for target proteins. DAB was observed and photographed under a microscope (motic, DMB5-2231P1 type) after dark color development. The brown color was positive. Image-Pro Plus 6.0 software was utilized for processing, and the integrated absorbance IA/area was used as the semiquantitative result of the detection index.

2.7. Western Blot Analysis. Liver or small intestine samples were ground and mixed with 1 mL of total protein extract to homogenize before centrifugation at 9000 rpm for 10 minutes to assess protein concentration. After boiling the protein for 3 minutes to denature it, the samples were put in a specified sequence for electrophoresis. When the bromophenol blue migrated to the bottom 0.5 cm of the separation gel, the gel glass plate was removed, and the polyvinylidene fluoride (PVDF) membrane was transferred. The electrophoresis was completed. After that, the electrotransfer membrane was blocked with 5% nonfat dry milk (PBS). At 4°C overnight, primary antibodies TLR4 (Ptgcn, 19811-1-AP), NF-κB p65 (Ptgcn, 10745-1-AP), MyD88 (Ptgcn, 23230-1-AP), IL-1β (CST, #12242), IL-6 (CST, #12912), TNF-α (Ptgcn, 60291-1-Ig), and β-actin (Ptgcn, 66009-1-Ig) were mixed with 1 mL of enzyme-labeled secondary antibody. After washing the membrane with 2-3 mL of PBST, develop it with ECL reagent and assess the gray value of each band using Image-Pro Plus 6.0 software. The relative protein expression is determined by the ratio of the target protein band to β-actin.

2.8. Statistical Analysis. GraphPad Prism 8.0 was utilized for the analysis. The experimental data were presented as the mean ± standard deviation ($x \pm s$), and they were tested for normality and variance homogeneity. When comparing two samples, the t-test was used, and when comparing multiple groups, one-way ANOVA was used. $P < 0.05$ indicating a statistically significant difference.

3. Results

3.1. TAK-242 Effect of Reducing HF in HF Rats. The hepatocytes of the rats in the control group were neatly arranged, with a clear structure, no degeneration or necrosis, no congestion in the hepatic sinus, and also no inflammatory cell infiltrate or fibrotic tissue proliferation, while in the model group, a considerable number of foam cells were found in the tissue of the rats and infiltration with a small number of lymphocytes and hyperplasia of connective tissue around a large number of venous vessels, accompanied by punctate necrosis of hepatocytes, nuclear fragmentation or lysis, enhanced eosinophilic cytoplasm, and rare bile duct hyperplasia. The TAK-242 group's liver tissue structure improved to varying degrees, connective tissue hyperplasia was greatly decreased, and the fibrous septum was significantly reduced, as shown in Figure 2. Furthermore, serum levels of AST, ALT, DBIL, and TBIL in the model group were significantly higher than in the control group ($P < 0.01$). The levels of AST, ALT, DBIL, and TBIL in serum of the TAK-242 group were significantly lower ($P < 0.01$) than those of the model group. The findings demonstrated that the CCL_4-induced rat hepatic fibrosis model was effectively created.

3.2. TAK-242 Effect on Liver Inflammation in HF Rats. HF upregulates inflammatory factors such as IL-1β, IL-6, and TNF-α in the liver. Compared to the control group, the model group had significantly higher levels of IL-1β, IL-6, and TNF-α secretion and expression. In comparison to the model group, the TAK-242 group significantly reduced serum levels of IL-1β, IL-6, and TNF-α. Meanwhile, the protein and mRNA expressions of IL-1β, IL-6, and TNF-α were significantly lower in the TAK-242 group's liver tissue, Figures 3(a)–3(h). These findings suggested that TAK-242 might reduce the inflammatory response in HF rats.

3.3. TAK-242 Effect in Intestinal Barrier Function of HF Rats. Claudin-1 and ZO-1 are two typical tight junction proteins that play important roles in the intestinal epithelium's tight junctions and permeability. The small intestine tissue cells in the control group's rats were well arranged, and there were no aberrant intestinal villi or cell infiltration. The mucosal layer of the small intestine tissue of the model group rats revealed a lot of epithelial edema, loose cytoplasm, and light staining, with dispersed lymphocyte infiltration, and a minor quantity of the epithelium was necrotic and shed, with condensed and stained nuclei. There were multiple mucosal layers and moderate edema of intestinal villi in the TAK-242 group's intestinal tissue, and the epithelium was separated from the lamina propria. The small intestine tissue of the TAK-242 group was improved to

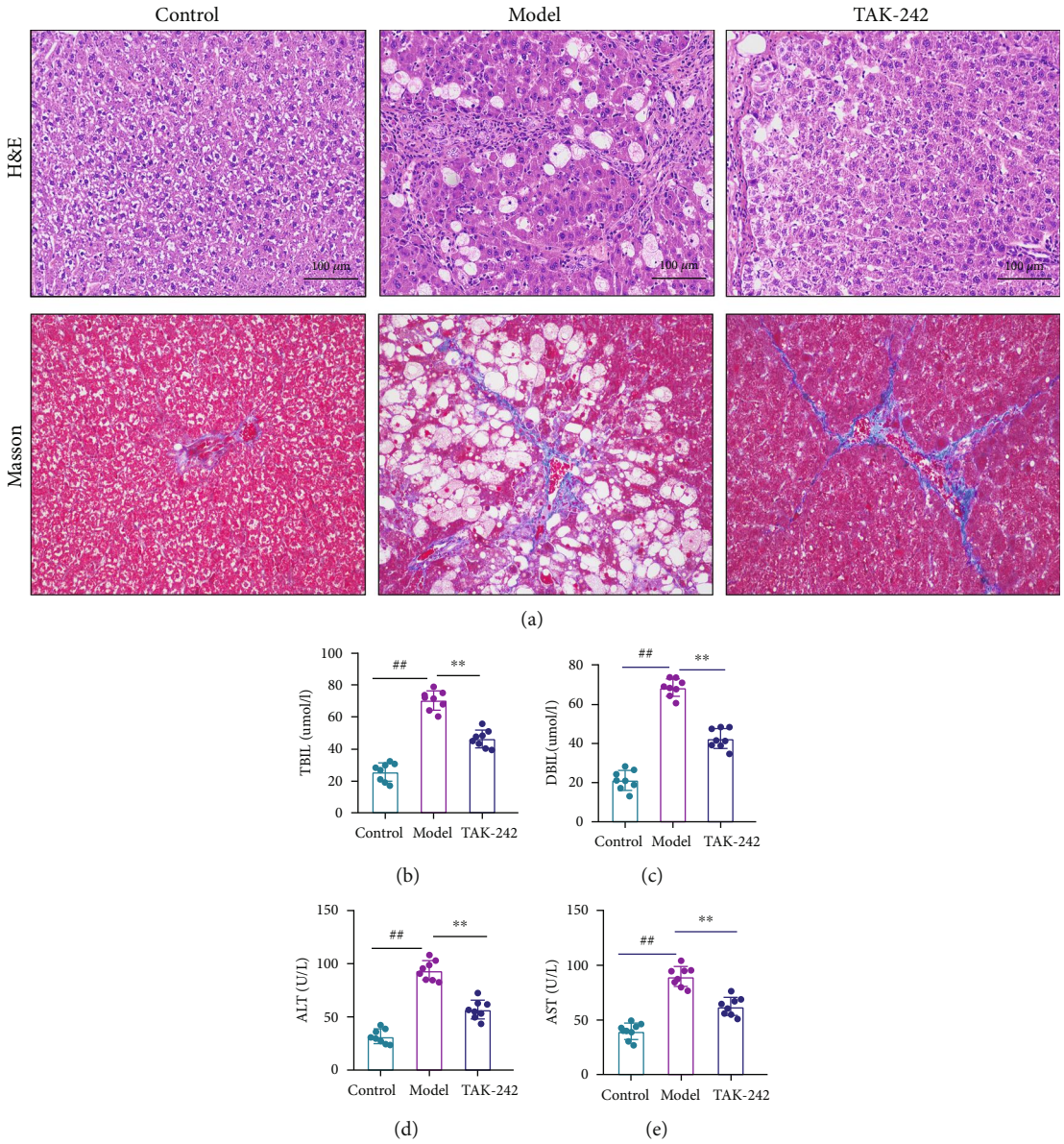

Figure 2: TAK-242 can alleviate HF-induced hepatic fibrosis in rat liver tissue. (a) Hematoxylin-eosin (H&E) staining and Masson staining of liver tissue (magnification, ×200). (b) Serum TBIL (μmol/L). (c) Serum DBIL (μmol/L). (d) Serum ALT (U/L). (e) Serum AST (U/L). $n = 8$. Compared with the control group, $^{\#\#}P < 0.01$; compared with the model group, $^{*}P < 0.05$ and $^{**}P < 0.01$. (b–e) Green, control; red, model; blue, TAK-242.

varying degrees as compared to the model group, and intestinal villus edema and lymphatic infiltration were dramatically reduced, Figure 4(a). In terms of mRNA and protein levels, claudin-1 and ZO-1 in the model group were significantly lower than those in the control group. In contrast, claudin-1 and ZO-1 in the TAK-242 group were significantly lower than in the model group (Figures 4(d) and 4(e)).

3.4. TAK-242 Can Reduce Intestinal Inflammation in HF Rats. When the intestinal epithelium's tight junctions and permeability are destroyed, the mucosal barrier function of the intestinal barrier is weakened, resulting in an inflammatory reaction in the intestinal tract that acts on the liver via the portal venous system and aggravates the pathological changes in the liver tissue. The expressions of IL-1β, IL-6, and TNF-α in the intestinal wall were measured to assess the influence of HF on the intestine. The mRNA and protein levels of IL-1β, IL-6, and TNF-α secretion and expression levels were increased significantly in the model group compared to the control group, while the secretion and expression levels of IL-1β, IL-6, and TNF-α in the TAK-242 group were significantly decreased compared with those in the model group (Figures 5(a)–5(e)). These results indicate that TAK-242 may reduce intestinal inflammation caused by hepatic fibrosis.

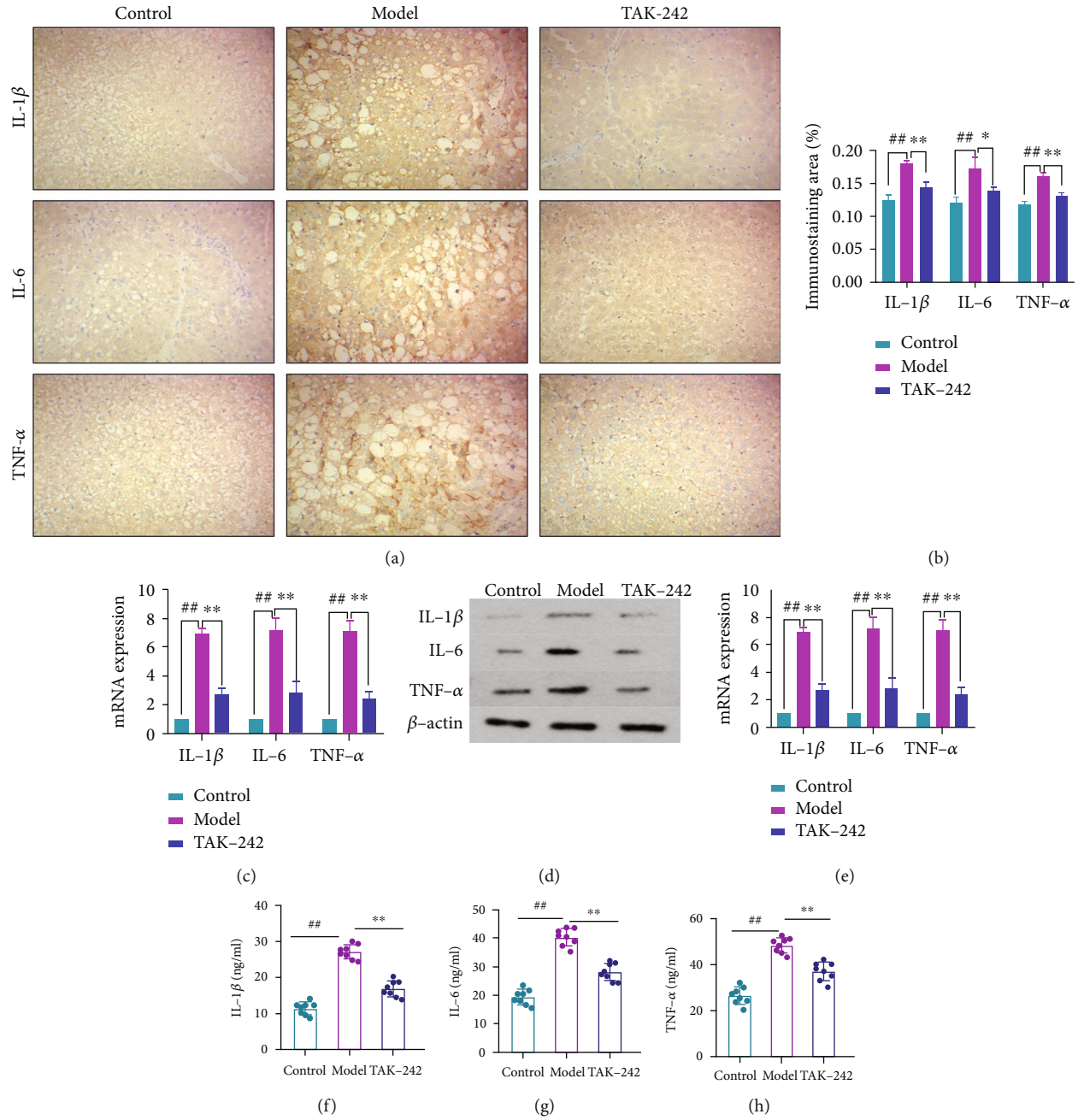

Figure 3: TAK-242 alleviated liver inflammation in HF rats. (a) IHC-stained liver sections (magnification ×200). (b) chromogenic intensity of proinflammatory cytokines. (c) RT-qPCR detection of hepatic proinflammatory cytokine expression level. (d, e) Western blot detection of hepatic proinflammatory cytokine protein expression. (f) Serum IL-1β (ng/mL). (g) Serum IL-6 (ng/mL). (h) Serum TNF-α (ng/mL). $n = 8$. Compared with the control group, $^{\#\#}P < 0.01$; compared with model group, $^{*}P < 0.05$ and $^{**}P < 0.01$. (f–h) Green, control; red, model; blue, TAK-242.

3.5. Effects of TAK-242 on TLR4 Signaling Pathway Expression in HF Rats. TLR4 is the initial barrier to bacterial detection in the gut and is a key component of gut innate immunity. It functions as an immunological recognition receptor on the cell surface as well as an intracellular transmembrane signaling protein. The MyD88-dependent signaling pathway dominates the signal transduction process following TLR4 activation. By simultaneously activating different intracellular signal adaptor molecules, NF-κB downstream of the pathway is eventually activated to control the production of numerous inflammatory mediators. As a result, we identified key proteins associated with the TLR4 signaling pathway in the liver and small intestine tissue, respectively. The findings demonstrated that the model group had significantly greater TLR4, NF-κB, and MyD88 secretion and expression levels than the control group. TLR4, NF-κB, and MyD88 secretion and expression levels in the TAK-242 group were significantly lower than those in the model group, Figures 6(a)–6(j).

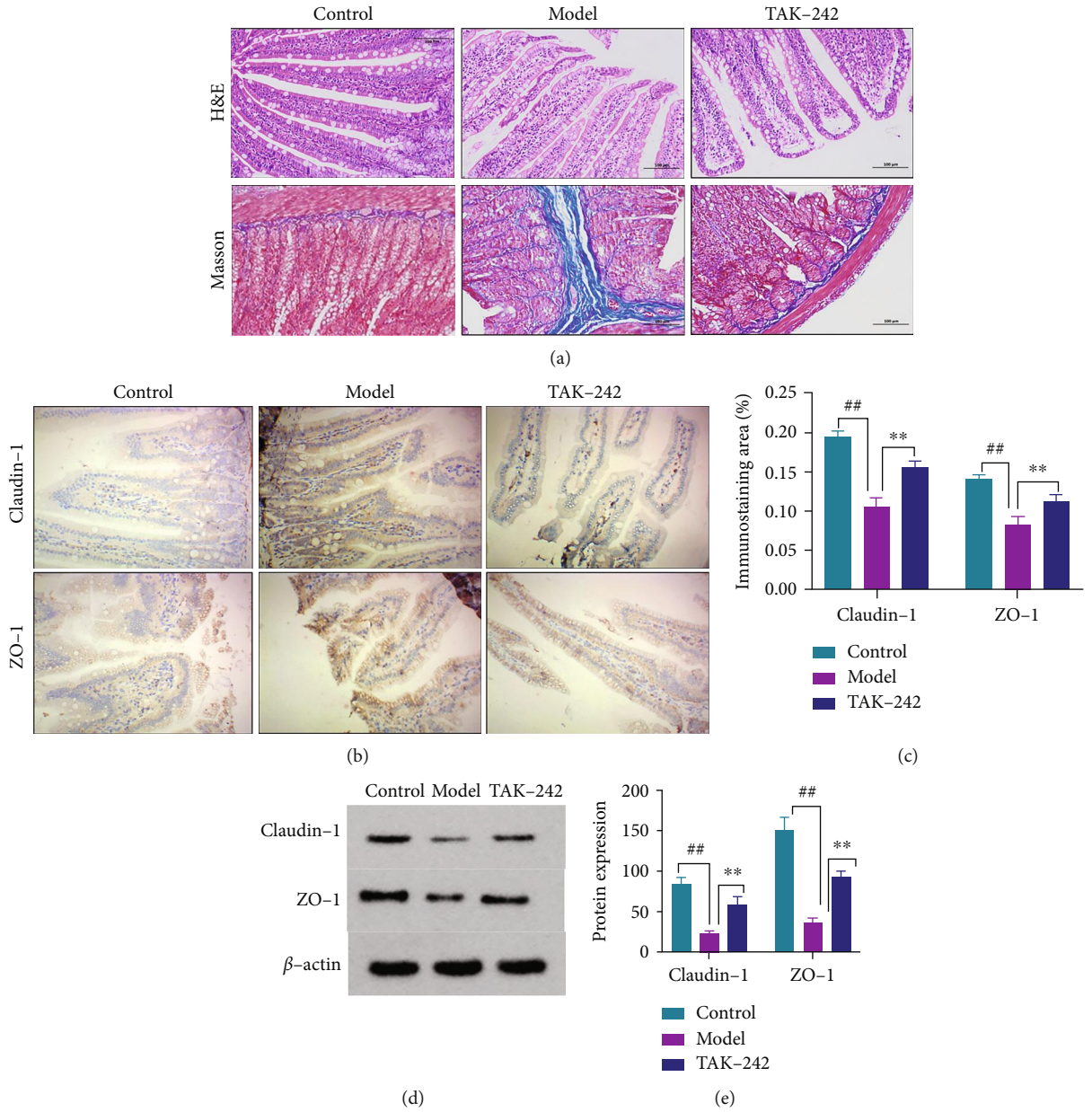

Figure 4: TAK-242 can regulate intestinal barrier function in HF rats. (a) Hematoxylin-eosin (H&E) staining of liver tissue, Masson staining (magnification, ×200). (b) IHC stained liver sections (magnification ×200). (c) Color intensity of intestinal wall permeability. (d, e) Western blot detection of intestinal wall tight junction protein expression. $n = 8$. Compared with the control group, $^{\#\#}P < 0.01$; compared with the model group, $^{*}P < 0.05$ and $^{**}P < 0.01$.

4. Discussion

The principal manifestation of liver fibrosis is an abnormal accumulation of extracellular matrix (ECM), which is typically regarded as an intermediate stage that may be cured or progress to cirrhosis and end-stage liver disease [28]. According to epidemiological data, more than one million individuals worldwide die from cirrhosis each year [29]. Cirrhosis is responsible for 9.2 fatalities per 100,000 people in the United States, according to epidemiological statistics from 2017 [30]. The burden of liver fibrosis raises not only the morbidity and mortality of end-stage liver disease but also the risk of extrahepatic disease. Modern research has established that the liver and the gut are not only physiologically connected not only in terms of structure (enterohepatic circulation) but also in terms of physiological functioning [31]. The findings of this investigation revealed that TAK-242 might inhibit the TLR4/MyD88/NF-κB signaling pathway through the liver-gut axis, hence curing HF.

Researchers have found that [32–34] ALT and AST are essential enzymes in the liver, and their levels are directly associated to the progression of liver fibrosis and inflammation, and when liver cells are injured, enzymes enter the bloodstream via the cells, and the function of the liver cells

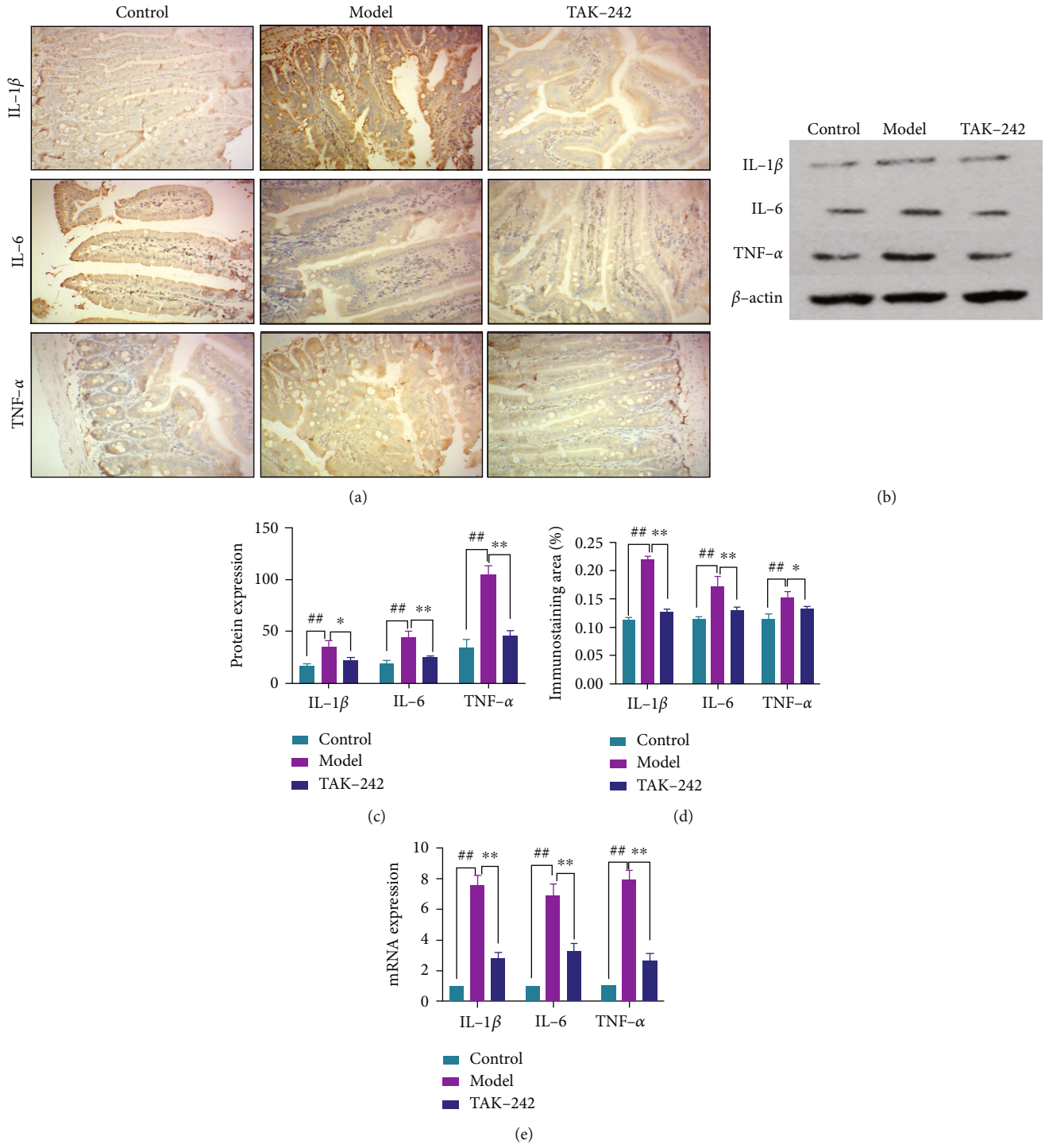

FIGURE 5: TAK-242 alleviates intestinal inflammation in HF rats. (a) Liver sections stained by IHC (magnification ×200). (b, c) Protein expression of intestinal proinflammatory cytokines detected by Western blot. (d) Color intensity of proinflammatory factors. (e) Expression of intestinal proinflammatory cytokines detected by RT-qPCR. $n = 8$. Compared with the control group, $^{\#\#}P < 0.01$; compared with the model group, $^{*}P < 0.05$ and $^{**}P < 0.01$.

to convert bilirubin is compromised. Inflammation in liver tissue destroys the capillary bile duct and impairs direct bilirubin excretion, resulting in elevated AST, ALT, TBIL, and DBIL levels. Our findings also revealed that serum AST, ALT, TBIL, and DBIL levels were greater in the model group than in the control group and that serum AST, ALT, TBIL, and DBIL levels could be significantly lowered following TAK-242 intervention. According to the pathological alterations in liver tissue, the model group had a high number of connective tissue and fibrous tissue hyperplasia and inflammatory cell infiltration, while the TAK-242 group's liver tissue improved to varied degrees. This result suggests

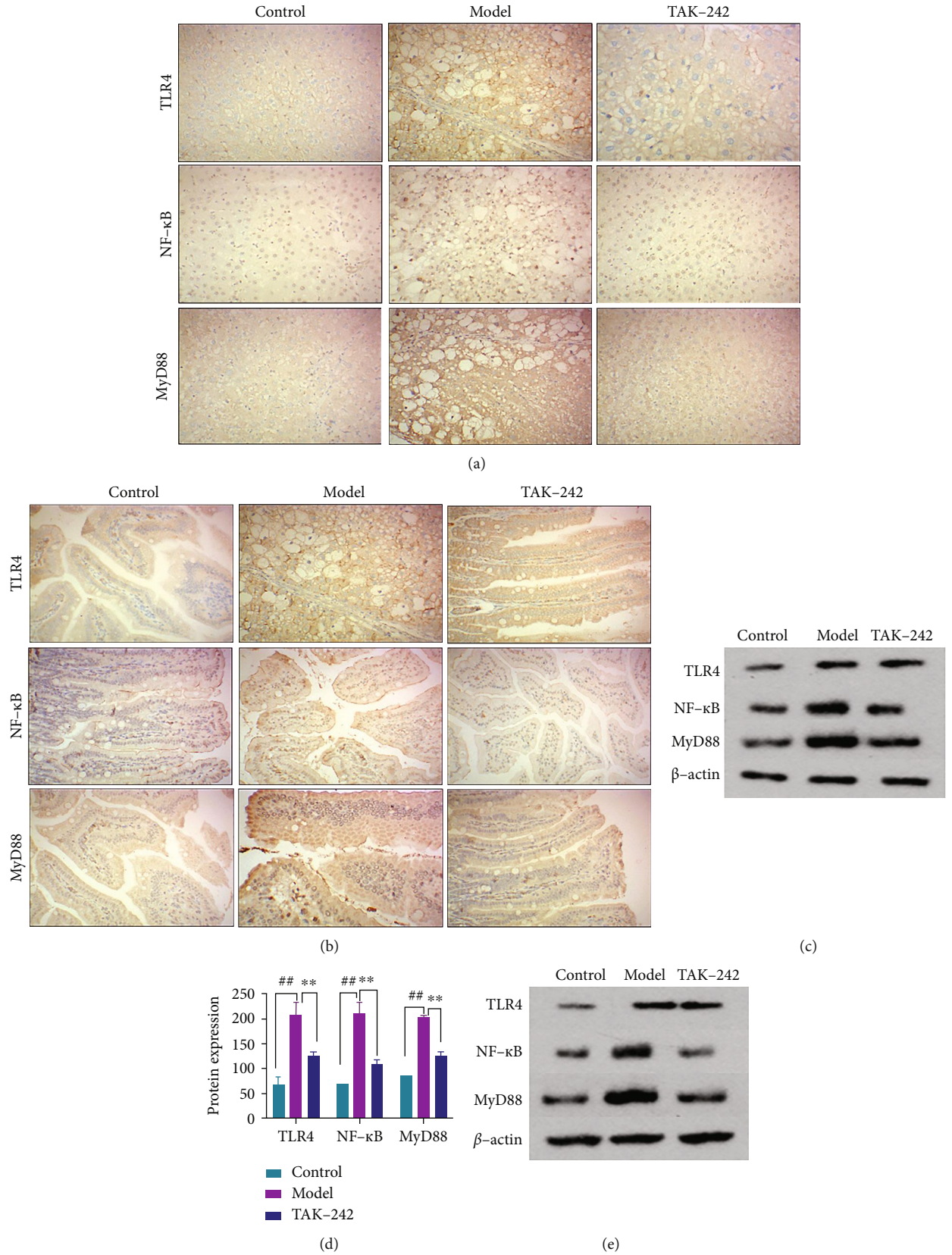

Figure 6: Continued.

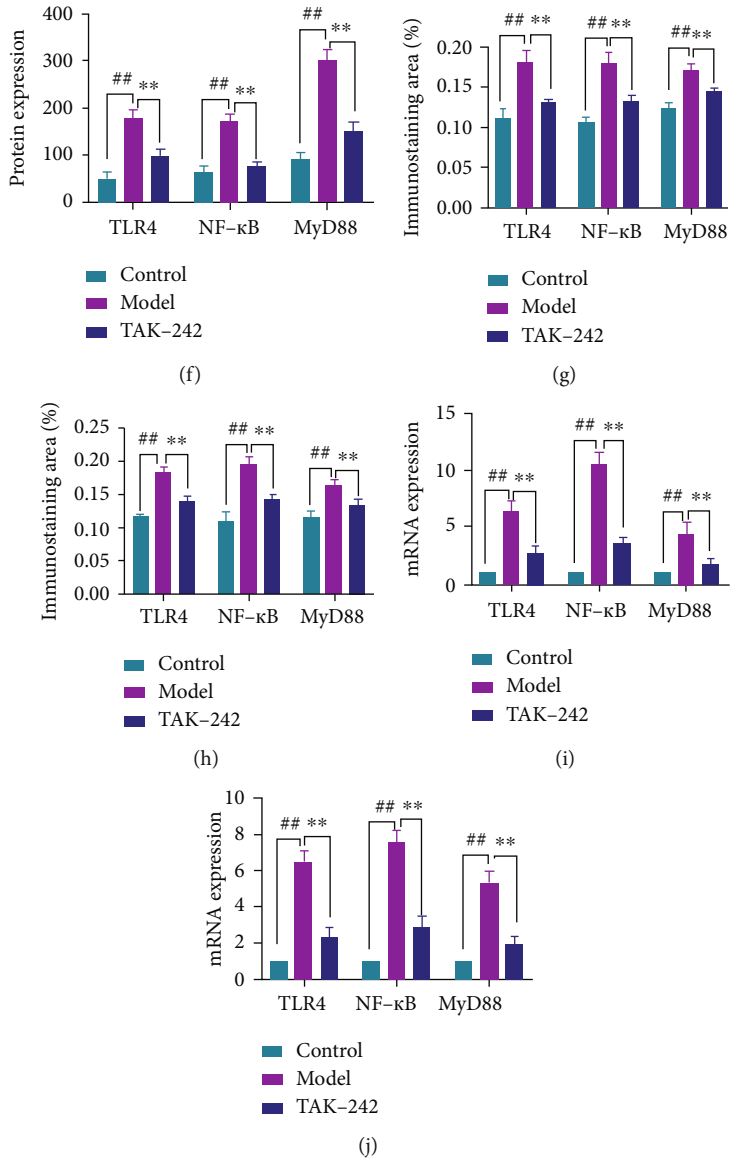

Figure 6: TAK-242's effect on TLR4 signaling pathway expression in HF rats. (a) IHC-stained liver section of liver tissue (magnification ×200). (b) IHC-stained intestinal section of intestinal tissue (magnification ×200). (c, d) Western blot detection of TLR4 signaling pathway protein expression in liver tissue. (e, f) Western blot detection of TLR4 signaling pathway-related protein expression in intestinal tissue. (g) Color intensity of TLR4 signaling pathway-related protein in liver tissue. (h) Color intensity of TLR4 signaling pathway-related protein in intestinal tissue. (i) RT-qPCR detection of TLR4 signaling pathway-related protein mRNA expression in liver tissue. (j) RT-qPCR detection of TLR4 signaling pathway-related protein mRNA expression in intestinal tissue. $n = 8$. Compared with the control group, $^{\#\#}P < 0.01$; compared with the model group, $^{*}P < 0.05$ and $^{**}P < 0.01$.

that TAK-242 may alleviate the HF damage induced by CCL_4.

Liu et al. [35] observed that bacterial translocation and elevated lipopolysaccharide levels in the gut stimulate TLR4 signaling and HSC activation in the liver. Meanwhile, claudins and occludins tight junctions play a vital role in the creation and maintenance of the intestinal epithelial barrier's integrity [36–38]. In our study, the intestinal mucosa tissues of HF rats in the model group were damaged, intestinal villi were diminished, and a considerable number of inflammatory cells were identified in the intestinal mucosa. Protein levels in the intestinal mucosa and villus were dramatically reduced after TAK-242 therapy. TAK-242 dramatically enhanced the protein levels of claudin-1 and ZO-1 in the small intestine of rats. TAK-242's antifibrosis activity in HF rats was suggested to be directly related to intestinal function.

In recent years, researchers have shown that inflammation is a major factor in the progression of liver fibrosis [39]. Liver injury may retain the active surface of HSCs and accelerate the migration of inflammatory cells to the injured liver, secreting a significant number of inflammatory mediators such as TNF-α, IL-6, and IL-1β to enhance the development of liver fibrosis [10–12, 40, 41]. TAK-242 was

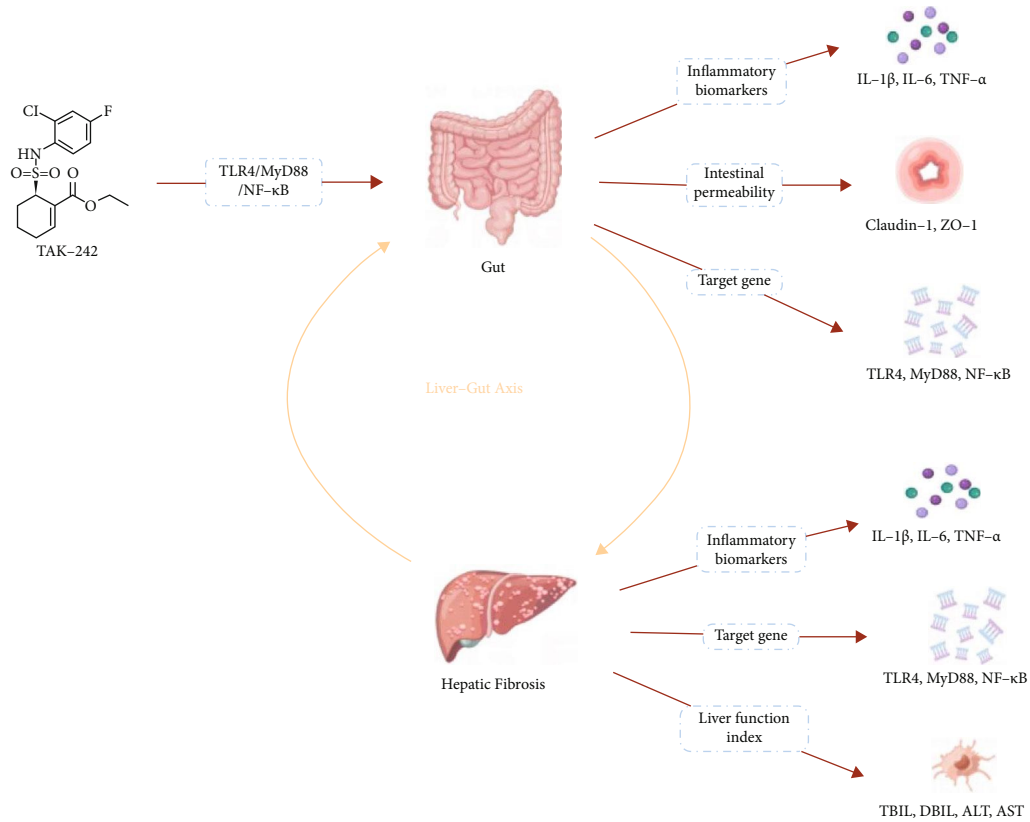

Figure 7: Flow chart of possible mechanisms of HF resistance in TAK-242, created using CmapTools.

also observed to reduce the incidence of liver inflammation and fibrosis in Hu et al.'s research [42]. Similarly, the current research found that TAK-242 lowered the levels of inflammatory factors IL-1β, IL-6, and TNF-α in the liver. Furthermore, following TAK-242 treatment, the contents of inflammatory components in the small intestine of rats reduced, indicating that control of TLR4 expression may not only suppress the inflammatory response in the liver of HF rats. It also reduced the inflammatory response mediated by intestinal mucosal barrier damage in HF rats. These results revealed that TAK-242's anti-HF action in rats was connected to improved liver inflammation through the liver-gut axis.

The TLR4 receptor is a pattern recognition receptor. Its primary ligands are PAMP (LPS and Gram-negative endotoxin) and DAMP, which include cell death products (mitotic nucleosomes, histones, and HMGB1) [43, 44]. The research by Wu et al. [45] revealed that this membrane receptor was expressed on a wide range of nonsubstantial and substantial cells, including hepatocytes and hepatic stellate cells. Following ligand binding, the receptor dimers and recruits adaptor molecules, such as TIR-domain adaptor protein (TIRAP) MyD88 and TRIF-associated adaptor molecule (TRAM) TRIF, to create intracellular signaling complexes [45, 46]. MyD88-dependent signaling activates NF-κB, while the TRIF-dependent pathway modulates interferon regulators, resulting in cytokine and interferon production [13]. Previous research by Naihua Hu et al. [47, 48] has demonstrated that TLR4/MyD88/NF-κB may have an anti-inflammatory and hepatoprotective effect by decreasing ECM accumulation and inflammatory factor expression. It was consistent with our findings that TLR4, MyD88, and NF-κB levels in HF rat liver tissue were elevated, whereas TLR4, MyD88, and NF-κB levels were dramatically lowered following TAK-242 therapy. TLR4 is the initial barrier to bacterial detection in the gut and is a key aspect of gut innate immunity. It functions as a cell surface immunological recognition receptor and an intracellular transmembrane signaling protein. Furthermore, TLR4, MyD88, and NF-κB levels in the small intestine of HF rats were significantly reduced after TAK-242 intervention, implying that TAK-242 improvement in HF rats may play a role by inhibiting the inflammatory response mediated by the TLR4/MyD88/NF-κB signaling pathway via the liver-gut axis.

Furthermore, changes in intestinal flora may help to explain the therapeutic mechanism; however, no research was conducted for this paper. As a result, the particular mechanism of TAK-242's therapeutic action on HF through the liver-gut axis requires additional investigation. Nonetheless, there is no doubting that this research has demonstrated the critical function of TAK-242 in the management of hepatic fibrosis. Nonetheless, this study has described an important role for TAK-242 in the treatment of liver fibrosis. The creation process using CmapTools is shown in Figure 7 [49].

5. Conclusion

In summary, this research illustrates that TAK-242 is implicated in CCL_4-induced HF and inflammatory factor release in HF rats. TAK-242's anti-Hf effect is most likely achieved by liver-gut axis suppression of inflammation through the TLR4/MyD88/NF-κB signaling pathway.

Abbreviation

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
DBIL: Direct bilirubin
TBIL: Total bilirubin
IL-1β: Interleukin-1β
IL-6: Interleukin-6
TNF-α: Tumor necrosis factor alpha
TLR4: Toll-like receptor 4
MyD88: Myeloid differentiation factor 88
NF-κB: Nuclear factor kappa B
ZO-1: Membrane protein
Claudin-1: Transmembrane proteins.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors state that they have no conflicts of interest.

Authors' Contributions

Sujie Liu, Juan Wu, and Shumin Liu conceived and designed the experiments. Sujie Liu analyzed the data. Sujie Liu, Shadi A.D. Mohammed, and Jingbo Zhang performed the experiments and wrote the manuscript. Pingping Chen provided valuable suggestions for the research. All the authors have read and approved the final manuscript.

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Research Article

Comprehensive Bioinformatics Analysis of Toll-Like Receptors (TLRs) in Pan-Cancer

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Background. To conduct a comprehensive bioinformatics analysis on the transcriptome signatures of Toll-like receptors (TLRs) in pan-cancer. *Materials and methods*. A total of 11,057 tissues consisting of 33 types of carcinoma in The Cancer Genome Atlas (TCGA) were retrieved, and then we further explored the correlation between TLRs' expression with tumorigenesis, immune infiltration, and drug sensitivity. We conducted a comprehensive bioinformatics analysis on TLR1 to 10 in pan-cancer, including differential expression analysis between normal and tumor tissues, differential immune subtype correlation, survival analysis, tumor immune infiltration estimating, stemness indices correlation, and drug responses correlation. *Results*. TLR2 was highly expressed in most types of tumors. TLR9 was hardly expressed compared to other TLR genes, which lead to TLR9 showing less correlation with both immune-estimate scores and stromal-estimate scores. All the TLRs were related with immune subtype of tumor samples that all of them were differentially expressed in differential immune subtype samples. The expression of TLRs was positively related with immune-estimate scores and stromal-estimate scores in almost all types of tumor. The expression of TLRs was negatively correlated with mRNA expression-based stemness scores (RNAss) in nearly almost type of tumors except kidney renal clear cell carcinoma (KIRC) and also negatively correlated with DNA methylation-based stemness scores (DNAss) in many types of tumors except adrenocortical carcinoma (ACC), cholangiocarcinoma (CHOL), KIRC, acute myeloid leukemia (LAML), low-grade glioma (LGG), testicular germ cell tumors (TGCT), thyroid carcinoma (THCA), thymoma (THYM), and uveal melanoma (UVM). The expression of TLR9 was significantly positively correlated with the drug sensitivity of fluphenazine, alectinib, carmustine, and 7−hydroxystaurosporine. TLR7 was significantly positively correlated with the drug sensitivity of alectinib. *Conclusions*. Our study reveals the significant role of TLRs family in pan-cancer and provides potential therapeutic strategies of cancer.

1. Introduction

Toll-like receptors (TLRs) are a family of transmembrane pattern recognition receptors that play essential roles in innate immunity for the detection of and defense against microbial pathogens [1]. TLRs are the first-line protective immune sentries that can recognize pathogen-associated molecular patterns (PAMPs), which typically include unmethylated double-stranded DNA (CpG), single-stranded RNA (ssRNA), lipoproteins, lipopolysaccharide (LPS), and flagellin [2]. They have been widely studied as the main mediators of innate immunity in animals, from insects to humans [3–5]. The discovery of TLRs as components that recognize the conserved structures in pathogens has greatly promoted the understanding of how the body perceives pathogen invasion, triggers innate immune responses, and initiates antigen-specific adaptive immunity [6].

It was reported that Drosophila strains with mutants of the Toll gene were highly susceptible to fungal infection, which was the first indication of the innate immune function of TLRs [7]. A human Toll homologue, now called TLR4,

was then identified [8]. Currently, a total of 10 TLR family members have been identified in humans, and at least 13 have been discovered in mice. These are usually expressed by various immune cells, such as dendritic cells (DCs), macrophages, T-cell subsets, and B-cells. They are also expressed in nonimmune cells (e.g., epithelial cells and fibroblasts) in humans [9]. All TLRs include an N-terminal domain characterized by multiple leucine-rich repeats and a carboxyl-terminal TIR domain that interacts with TIR-containing adapters. Nucleic acid-sensing TLRs (TLR3, TLR7, TLR8, and TLR9) are located in the endoplasmic chamber, whereas the remaining TLRs are present on the plasma membrane [10, 11].

In recent years, TLRs have gained great interest in cancer research because of their role in tumor progression, and many therapeutic interventions for TLR have been developed or studied. Some studies have explored in detail the role of TLR regulation in cancer development [12–14]. Compared to that in normal patients, the expression of TLR1, 2, 4, and 8 mRNA was increased in patients with colorectal cancer [15]. TLRs have also been associated with prostate cancer, but they may be a double-edged sword in prostate tumorigenesis because they can both promote malignant transformation of epithelial cells thereby enhancing tumor growth and induce apoptosis, thus, inhibiting tumor progression [16]. In addition, the regulation of TLRs not only increases the susceptibility to infection from some microorganisms but also contributes to the development of cancer by altering the microbiota resulting in inflammation [17]. On one hand, TLRs play an essential role in tumor immunity by activating a variety of cells, such as DCs, T-cell subsets, and even tumor cells; on the other hand, the activation of TLRs can also lead to inflammation that results in tumor promotion [18].

However, the characteristics of TLRs differ, and different homologous types may have different effects on different tumor types. In addition, to date, no bioinformatics study has systematically investigated the transcriptional levels of each TLR across multiple cancers. Therefore, it is of great significance to study the expression patterns of TLRs in cancer tissues and to develop potential TLR-targeted drugs for treatment of tumors with differentially expressed TLRs. In this study, we analyzed the expression characteristics of TLR1 to TLR 10 in various cancer tissues using a variety of bioinformatics methods, comprehensively analyzed TLRs, and found that the transcriptional levels of TLRs were associated with stemness, tumor purity, and drug sensitivity in cancer tissues included in The Cancer Genome Atlas (TCGA).

2. Materials and Methods

2.1. Data Sources. The transcriptome profile, clinical phenotype information, survival information, immune subtype profile, and DNA and RNA stemness profiles of 33 types of tumors were downloaded from the Genomic Data Commons (GDC) TCGA sets or TCGA pan-cancer sets in the UCSC Xena database (http://xena.ucsc.edu/) on November 15, 2020. Transcriptome profiles containing both tumor and normal adjacent tumor (NAT) tissues yielded a total of 11,057 samples, coded as fragments per kilobase per million (FPKM).

2.2. Expression Status of TLRs across Multiple Cancer Types. We first extracted and visualized the pan-cancer expression of TLRs. We then selected the five most highly expressed TLRs for further differential expression analysis. We sorted the expression profiles for cancer types whose expression profiles retained the expression profile of NAT tissues, and they were BLCA, BRCA, CHOL, COAD, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, READ, STAD, THCA, and UCEC. We then extracted the expression of the 5 most highly expressed TLRs in these cancer types and performed differential expression analysis between tumors and NAT using the Wilcoxon test. In addition, for all the TLRs, we calculated the log2 fold change (logFC) of each TLR in these cancer types and presented it in a heatmap. Subsequently, we applied a correlation test to explore the coexpression of the 10 TLRs according to their expression profiles.

2.3. Prognostic Value of TLRs across Multiple Cancer Types. For each TLR gene and tumor type, we separately performed log-rank survival analysis (grouped by the medium expression of the TLR in each cancer type) and univariate Cox regression to explore the pan-cancer prognostic value of TLRs. We then visualized the survival curves with significant differences and drew a forest plot of the resulting hazard ratios (HRs) and their 95% confidence interval.

2.4. Immune Subtype Correlations, Stemness Indices Correlations, and Tumor Microenvironment (TME) Estimations. Based on the immune subtype profile of each TCGA sample downloaded from the UCSC Xena, we explored the differential expression status of TLRs in different immune subtypes using the Wilcoxon test. We further probed the correlation between the expression of TLRs and the stemness index of the tissue samples containing DNA methylation-based stemness scores (DNAss) and mRNA expression-based stemness scores (RNAss) across multiple cancer types using the Spearman's correlation test. In addition, we applied the ESTIMATE method to analyze the immune-estimate score and stromal-estimate score of each sample and then performed the Spearman's correlation test to examine the correlation between the expression of TLRs and these two scores.

2.5. Drug Sensitivity Analysis of TLRs across Multiple Cancer Types. Data including both expression of TLRs and drug sensitivity were retrieved from the CellMiner database ((https://discover.nci.nih.gov/cellminer/), which collects genomic and pharmacologic information for investigators to determine the correlation between gene expression and drug sensitivity in the NCI-60 cell line sets. Thus, we extracted the expression values of TLRs in NCI-60 cell lines and their corresponding drug sensitivities to different drugs and conducted a Pearson correlation test between the expression of TLRs and drug sensitivity to explore the drug sensitivity in patients.

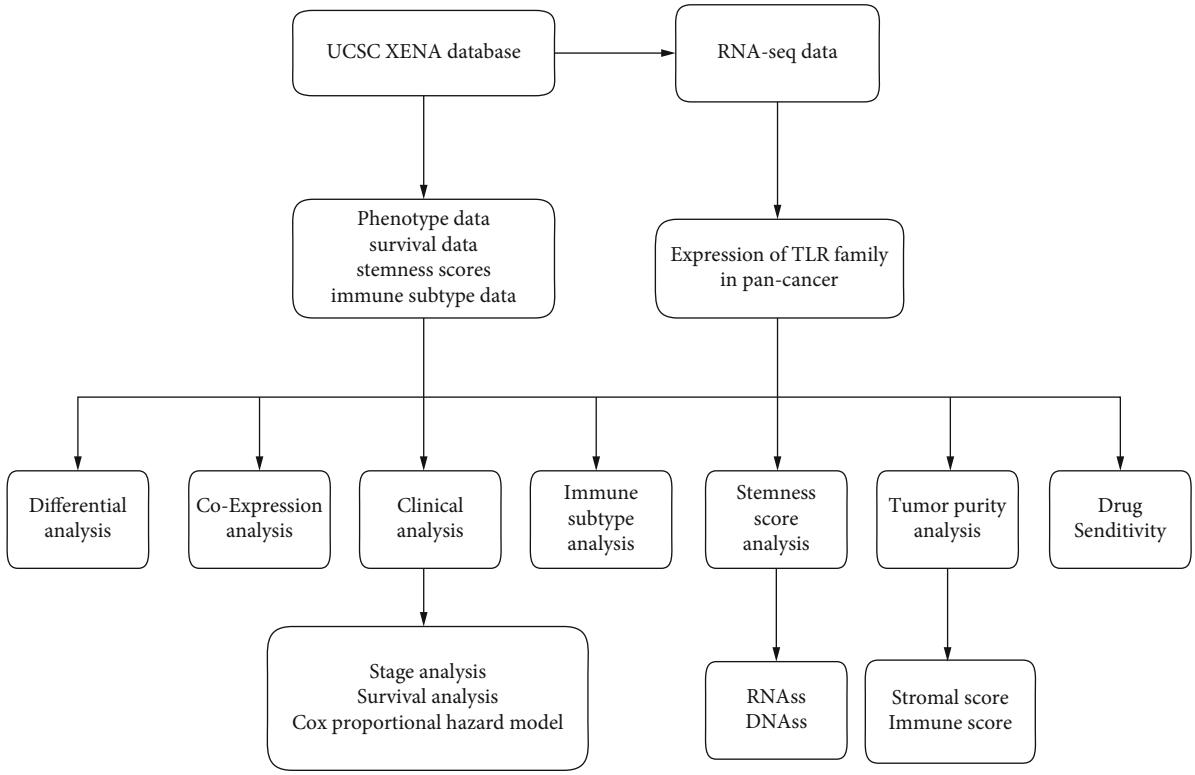


Figure 1: The study flow chart.

2.6. TLRs in KIRC. Finally, as TLR expression performed well in predicting the overall survival for KIRC, we further explored the significance of TLRs in KIRC. We separately investigated the differential expression of TLRs among different immune subtypes, the correlation between TLR expression and stemness indices, and the correlation between TLR expression and ESTIMATE scores in KIRC samples. In addition, we explored the differential expression status of TLRs between stages I and IV to determine whether TLRs could serve as biomarkers of survival and progression in KIRC.

2.7. Statistical Analysis. All statistical analyses were conducted using the R software (version 4.0.2). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Differential Expression Analysis of TLRs between Tumor and NAT Tissues. The flowchart of the study is summarized in Figure 1, and the abbreviations of the 33 tumor types in TCGA are provided in Table 1. The pan-cancer gene expression of TLR1 to TLR10 is displayed in Figure 2(a), and it seems that the expression of TLR9 was low compared to that of the other TLR genes. In addition, differential expression analysis with the Wilcoxon test was performed on the 10 TLR family genes between tumor and NAT tissues. Furthermore, the five most highly expressed genes, TLR1 to TLR5, were selected to show the differential expression status. TLR1 expression was significantly low in most types of cancers, except CHOL, GBM, and KIRC (Figure 2(b)). TLR2 was significantly expressed in most tumor types, except BRCA, LIHC, LUAD, LUSC, and PRAD (Figure 2(c)). TLR3 expression was significantly low in most type of tumors, except GBM and KIRC (Figure 2(d)). TLR4 expression was significantly low in most type of tumors, except GBM and KIRC (Figure 2(e)). TLR5 expression was significantly low in most type of tumors, except CHOL, GBM, and LIHC (Figure 2(f)).

3.2. Coexpression Analysis of TLRs across Multiple Cancer Types and Log-Rank Survival Analysis. More detailed information about the differential expression status, including log2FC, is shown in Figure 3(a). It was obvious that TLR2 was highly expressed in most types of cancer, and TLR family members were least expressed in LUSC and LUAD. In addition, coexpression analysis of TLRs suggested that all TLRs were positively correlated with each other, except TLR3, which was negatively correlated with TLR9 (Figure 3(b)). We then employed Kaplan–Meier methods to plot survival curves and performed a log-rank analysis to investigate the prognostic value of TLRs for the 33 TCGA cancers. The prognostic values of TLRs with cancer type and p value are shown in Table 2. We then selected KIRC to plot the survival curves for the four TLR genes with prognostic values for KIRC, TLR1 (Figure 3(c)), TLR3 (Figure 3(d)), TLR4 (Figure 3(e)), and TLR9 (Figure 3(f)). Among these, low expression of TLR1, TLR3, and TLR4 was significantly associated with poor overall survival, while high expression of TLR9 was significantly associated with poor overall survival in KIRC.

Table 1: Abbreviations of the 33 tumor types in TCGA.

Abbreviation	Tumor type
ACC	Adrenocortical carcinoma
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangiocarcinoma
COAD	Colon adenocarcinoma
DLBC	Lymphoid neoplasm diffuse large B-cell lymphoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
HNSC	Head and neck squamous cell carcinoma
KICH	Kidney chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LAML	Acute myeloid leukemia
LGG	Brain lower grade glioma
LIHC	Liver hepatocellular carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MESO	Mesothelioma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PCPG	Pheochromocytoma and paraganglioma
PRAD	Prostate adenocarcinoma
READ	Rectum adenocarcinoma
SARC	Sarcoma
SKCM	Skin cutaneous melanoma
STAD	Stomach adenocarcinoma
TGCT	Testicular germ cell tumors
THCA	Thyroid carcinoma
THYM	Thymoma
UCEC	Uterine corpus endometrial carcinoma
UCS	Uterine carcinosarcoma
UVM	Uveal melanoma

3.3. Cox Regression and Immune Subtype Analysis. Univariate Cox proportional hazard regression was performed to explore the prognostic values of TLRs for the 33 types of cancer. Genes were considered a risk factor if the HR was >1 or a protective factor if the HR was <1. According to the forest plot (Figure 4(a)), we found that TLRs play a complex role in cancer prognosis, which is risky in some types of tumors but protective in the remaining types of tumors. In addition, we performed a Kruskal test on the expression of TLRs in the six immune subtypes across the 33 TCGA cancer types (Figure 4(b)). Interestingly, all TLRs were differentially expressed in the different immune subtype samples. Among them, TLR1, TLR2, TLR3, TLR5, TLR6, TLR7, and TLR8 showed the highest expression in C6 immune subtype samples, whereas TLR4 and TLR10 showed the highest expression in the C5 immune subtype.

3.4. TLRs and TME across Multiple Cancer Types. Immune-estimate scores and stromal-estimate scores of samples were calculated using the R package "ESTIMATE" [19], and Spearman's correlation test was used to explore the correlation between TLR expression and the TME. For the immune score, expression of TLRs was positively correlated with immune scores in almost all types of cancer, except TLR1 in UVM, TLR3, 4, and 5 in THYM, and TLR10 in DLBC (Figure 5(a)). In addition, for the stromal scores, the expression of TLRs was positively correlated with stromal scores in almost all types of cancer, except TLR1 in UVM and TLR3 in ACC, LAML, MESO, and READ (Figure 5(b)). TLR9 showed low correlation with both immune and stromal scores, which may be due to the low expression of TLR9 in all the tumor samples.

3.5. TLRs and Stemness Indices across Multiple Cancer Types. We downloaded the stemness indices for all the samples from the UCSC Xena database, which were calculated using the one-class logistic regression (OCLR) as proposed by Malta et al. [20]. Two types of stemness indices were assessed: DNAss and RNAss. Interestingly, the expression of TLRs was negatively correlated with RNAss in nearly all types of cancer, except KIRC (Figure 5(c)), and negatively correlated with DNAss in many types of cancer, except ACC, CHOL, KIRC, LAML, LGG, TGCT, THCA, THYM, and UVM (Figure 5(d)). Among the DNAss scores, nearly all TLRs, except for TLR7 and TLR9, were positively correlated with DNAss in THYM samples.

3.6. TLRs and Drug Responses across Multiple Cancer Types. The expression profile of NCI-60 cancer cell lines and their drug sensitivity were downloaded from the CellMiner database; the Pearson correlation test was then performed to further analyze the correlation between the expression and the response to 263 antineoplastic drugs. All results with significant correlation between TLRs and drug sensitivity are displayed in Supplementary Table (available here), and the 25 most significant results with the smallest p value are shown as scatter plots ranked by p value (Figure 5(e)). Among them, the five most significant correlations were as follows: the expression of TLR9 had a significant positive correlation with the response to fluphenazine (coefficient = 0.680, $p < 0.001$), alectinib (coefficient = 0.637, $p < 0.001$), carmustine (coefficient = 0.598, $p < 0.001$), and 7−hydroxystaurosporine (coefficient = 0.550, $p < 0.001$), while TLR7 had a significant positive correlation with alectinib (coefficient = 0.595, $p < 0.001$).

3.7. TLRs in KIRC. Finally, we explored TLRs in KIRC by comparing the transcriptional expression of TLRs at different stages of KIRC, comparing the differential expression of TLRs in different immune subtypes, and investigating the correlation between TLRs and stemness indices or tumor purity in KIRC. TLR2, TLR3, TLR4, and TLR10 were significantly differentially expressed between stages I and IV ($p < 0.05$) (Figure 6(a)), and TLR1, TLR3, TLR4, TLR7, TLR8, and TLR10 were significantly differentially expressed between the C1 and C6 immune subtypes

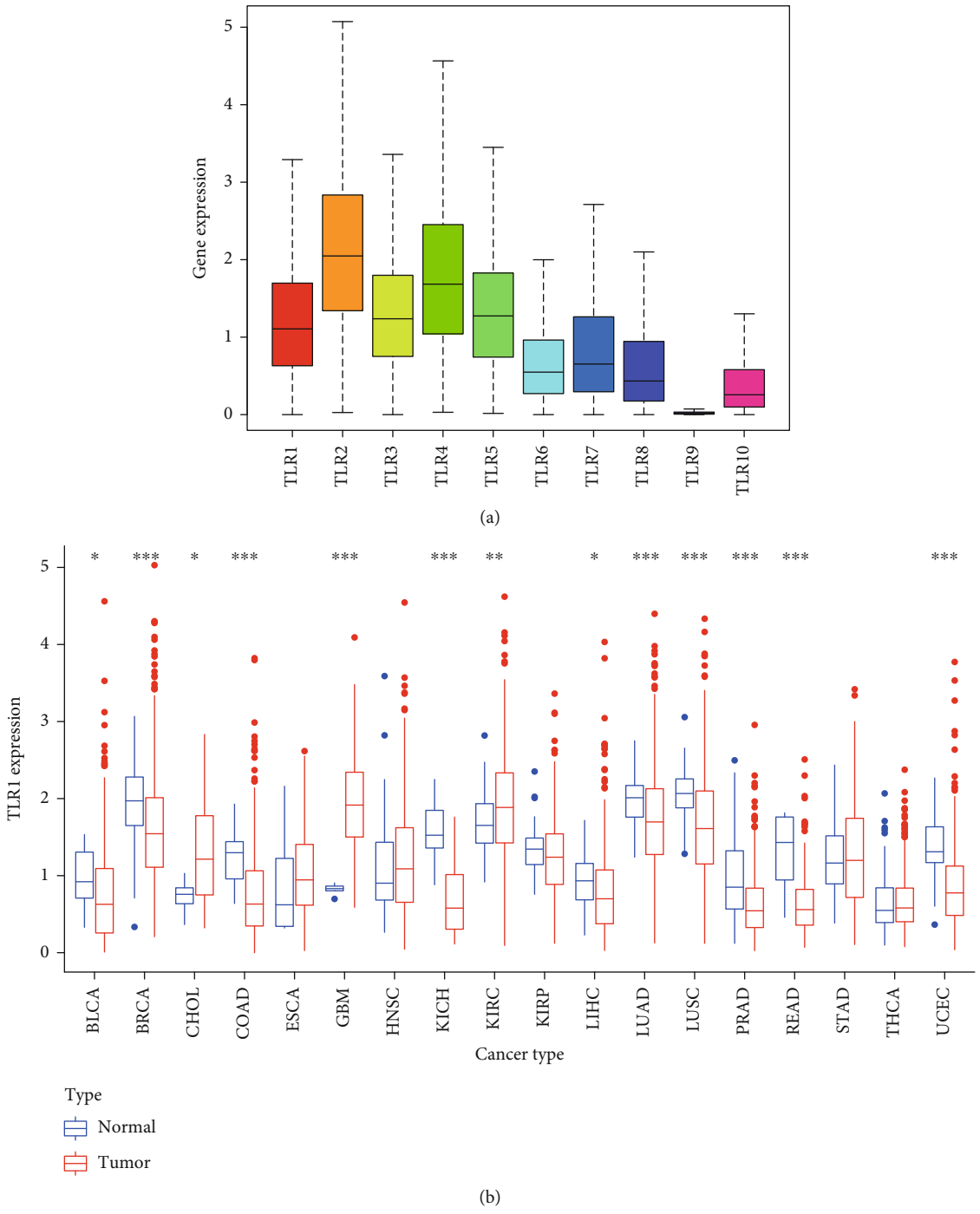
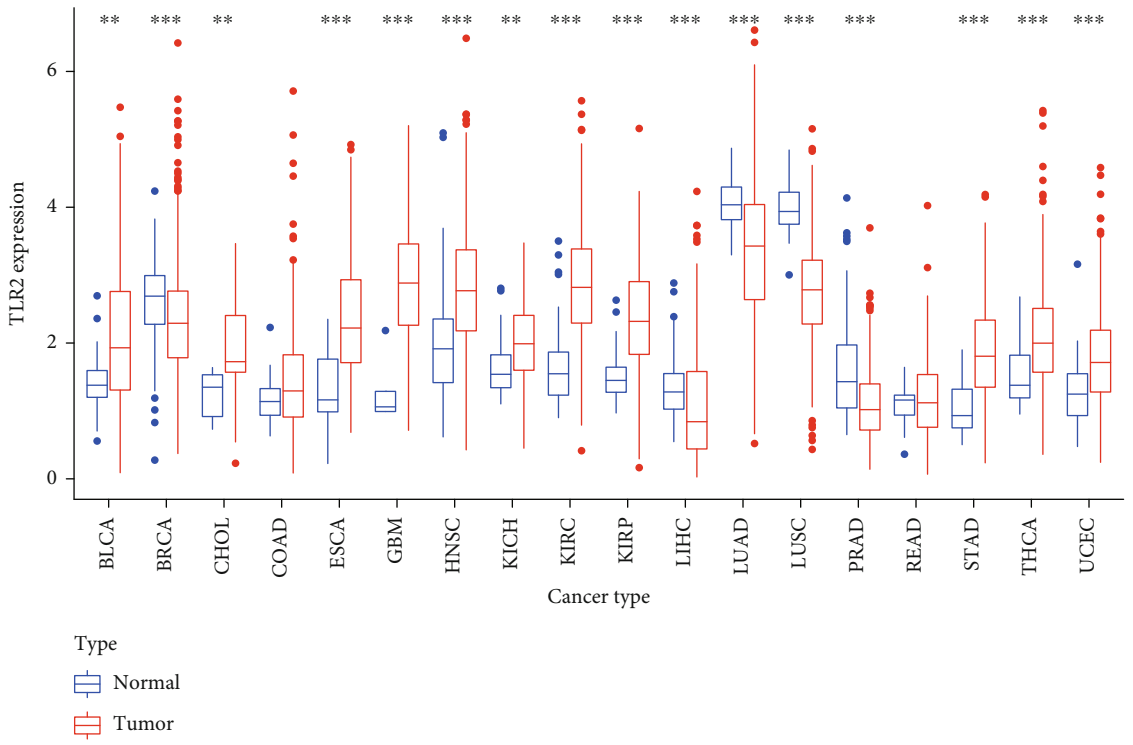


Figure 2: Continued.



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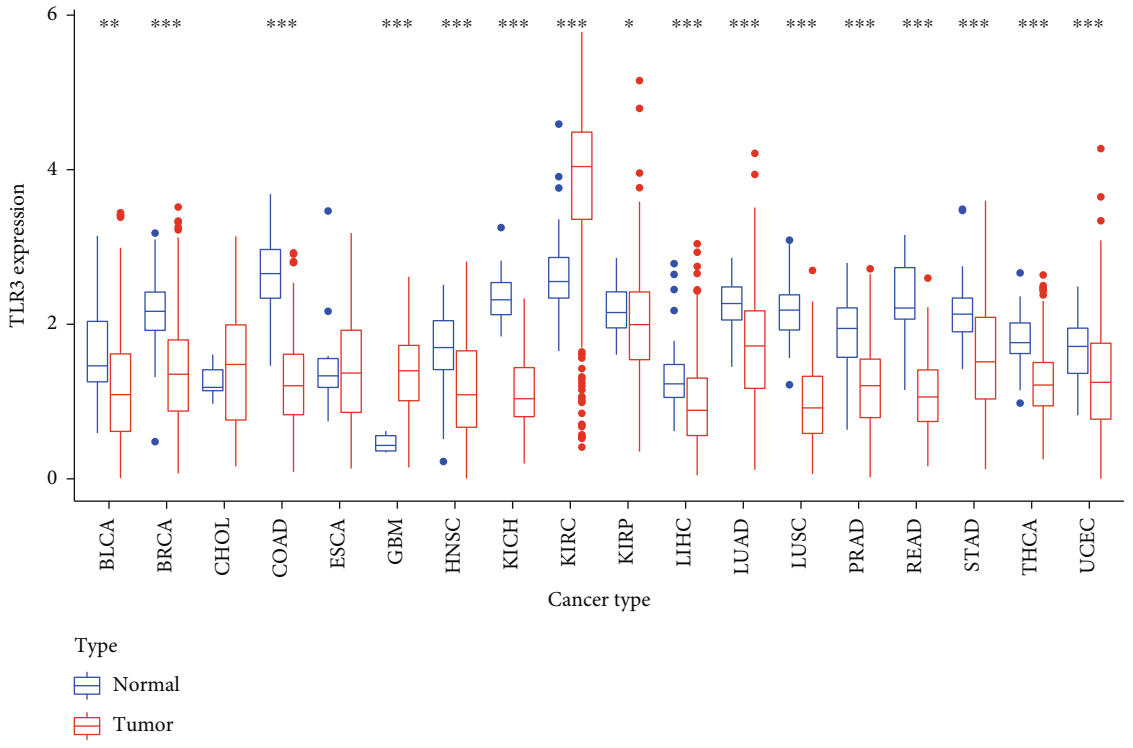

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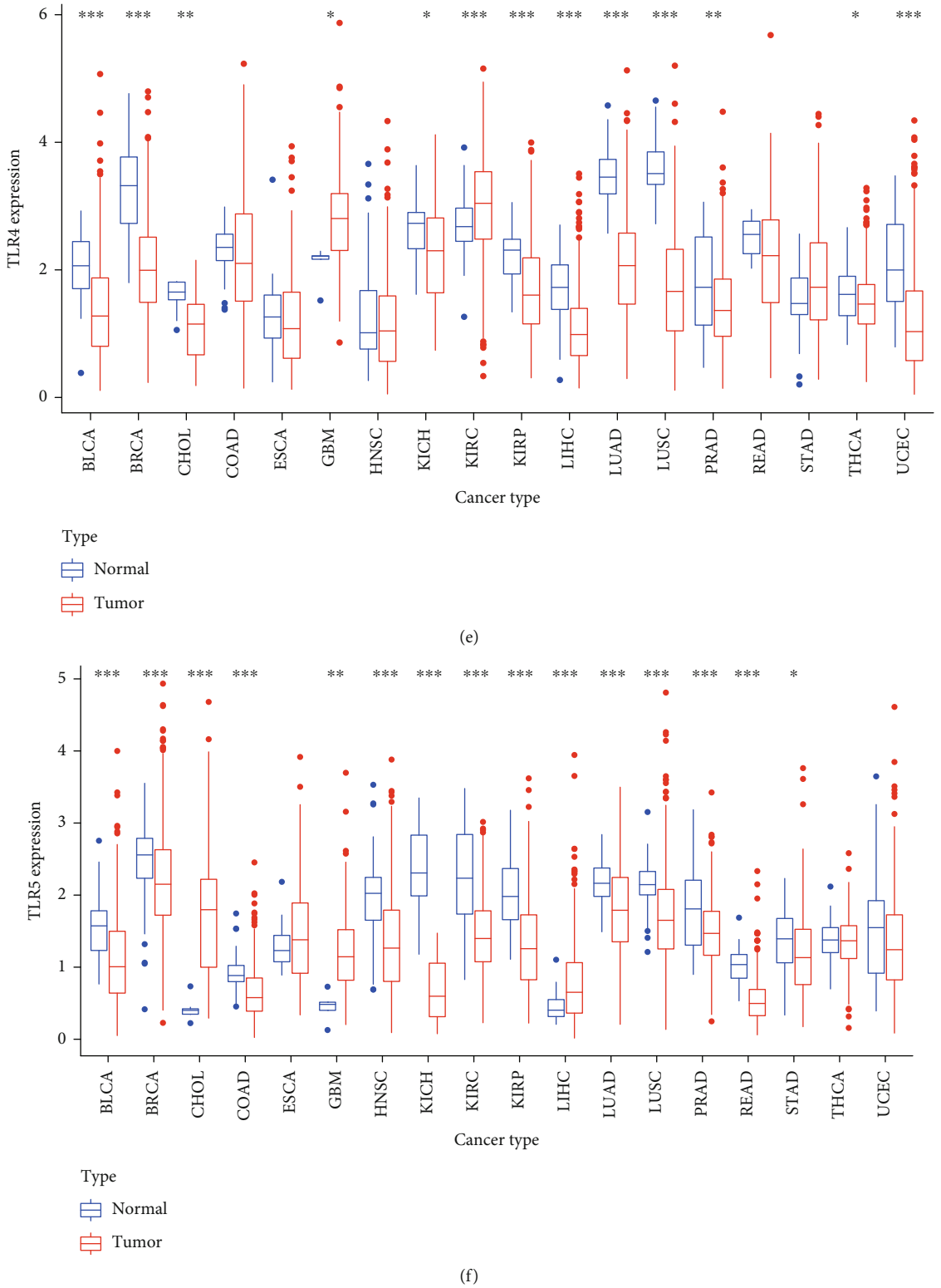
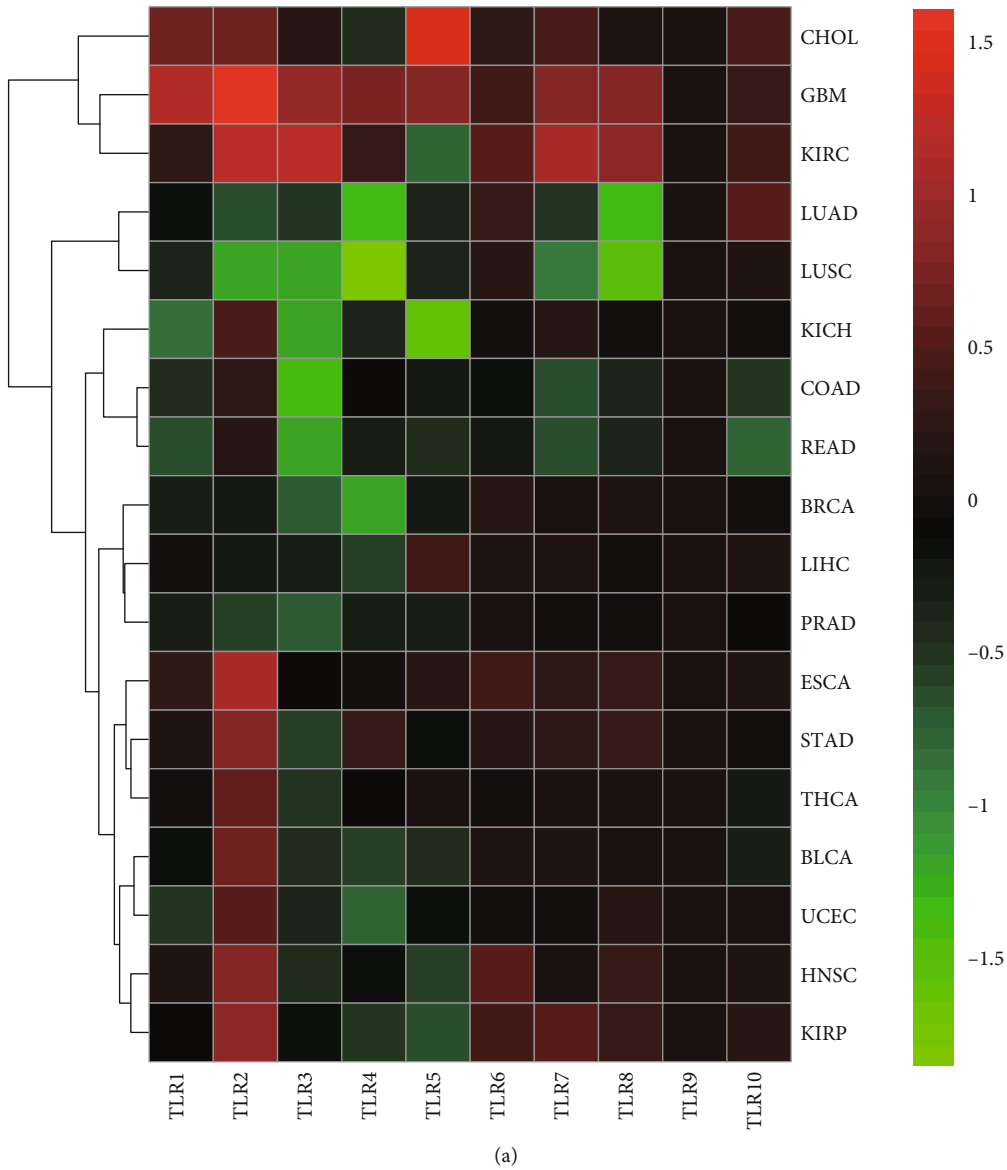


Figure 2: Expression status of TLRs. (a) Expression of TLRs in pan-cancer. (b) Differential expression of TLR1 in pan-cancer. (c) Differential expression of TLR2 in pan-cancer. (d) Differential expression of TLR3 in pan-cancer. (e) Differential expression of TLR4 in pan-cancer. (f) Differential expression of TLR5 in pan-cancer.



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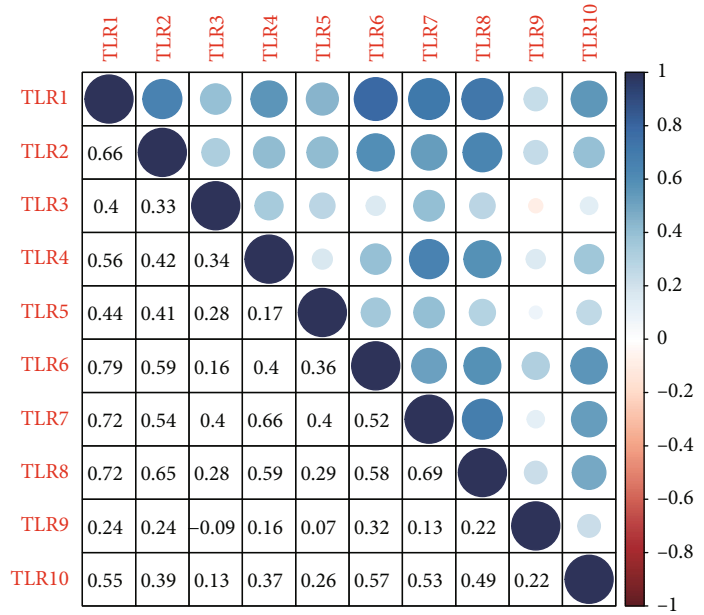

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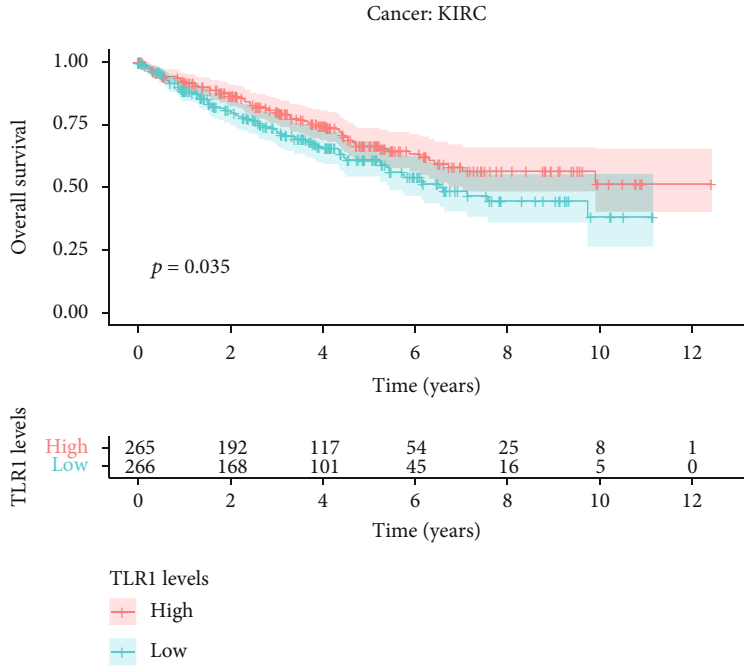

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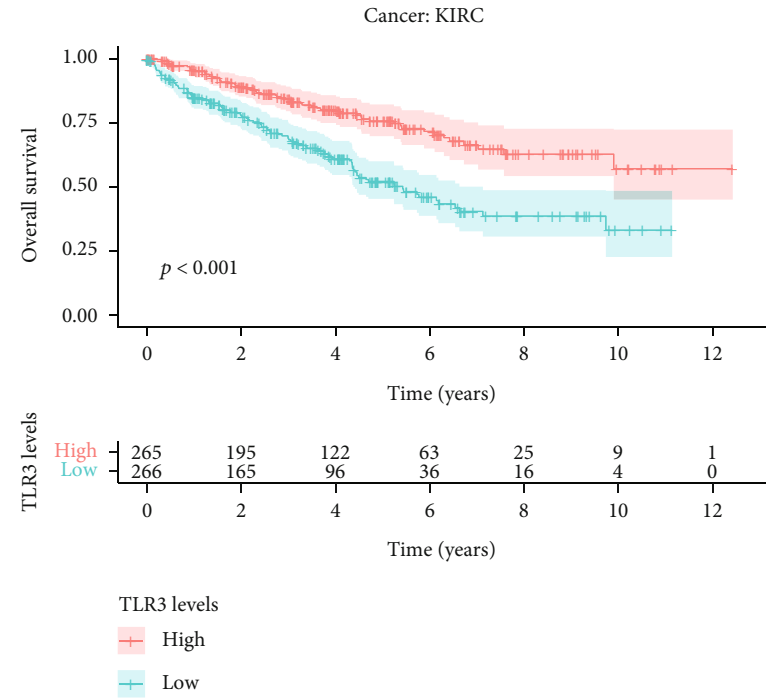

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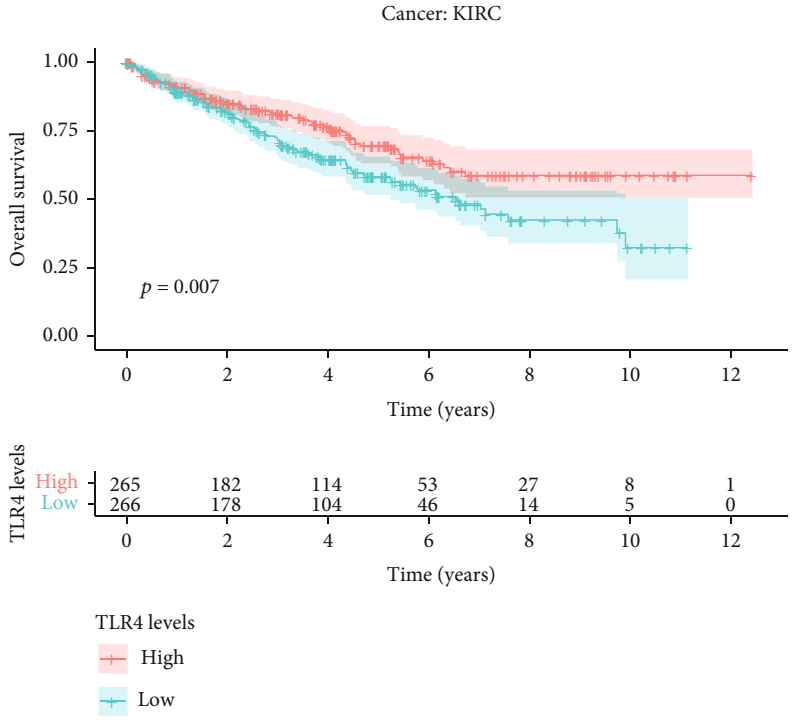

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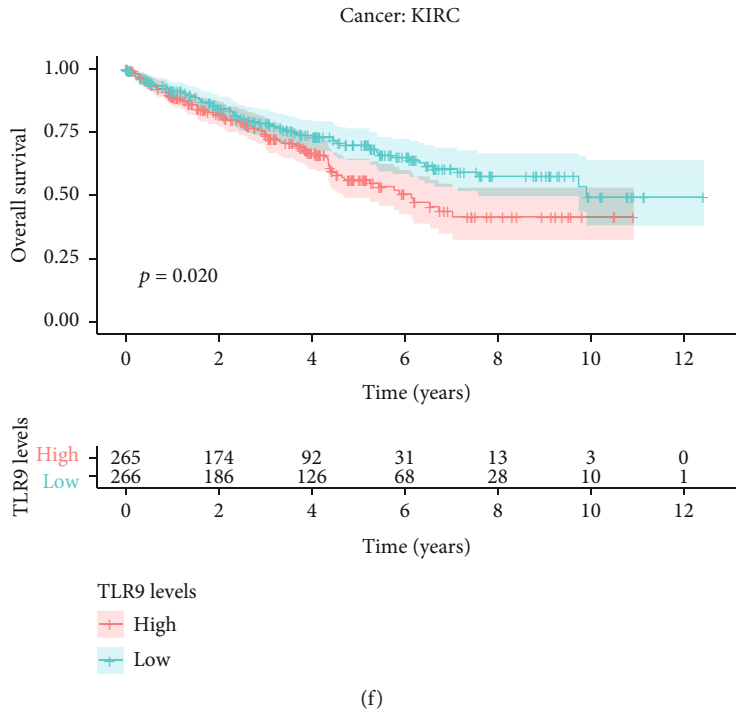


(f)

Figure 3: Coexpression of TLRs and survival curves in KIRC. (a) Differential expression status of TLR1 to TLR10 in pan-cancer. (b) Coexpression of TLRs in pan-cancer. (c) TLR1 as a candidate prognostic factor in KIRC. (d) TLR3 as a candidate prognostic factor in KIRC. (e) TLR4 as a candidate prognostic factor in KIRC. (f) TLR9 as a candidate prognostic factor in KIRC.

($p < 0.001$) (Figure 6(b)). For RNAss in the KIRC samples, TLR5 and TLR9 had significant negative correlations (correlation coefficient = −0.12, $p = 0.042$ and correlation coefficient = −0.23, $p < 0.001$, respectively), but TLR1, TLR2, and TLR3 had significant positive correlations (correlation coefficient = 0.11, $p = 0.048$; correlation coefficient = 0.14, $p = 0.014$; and correlation coefficient = 0.14; $p = 0.013$, respectively). For DNAss in the KIRC samples, it was interesting that all the TLRs were negatively correlated in KIRC patients, among which TLR1, TLR2, TLR6, TLR7, TLR8, and TLR10 were significant at $p < 0.05$. In addition, all the TLRs had significant positive correlations with the immune scores, stromal scores, and ESTIMATE scores. Among them, TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR10 were positively correlated with stromal scores ($p < 0.05$); TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10 were positively correlated with immune scores ($p < 0.05$); and TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR10 were positively correlated with the ESTIMATE scores ($p < 0.05$) (Figure 6(c)).

4. Discussion

Many studies have demonstrated that several cellular and molecular mechanisms can help tumors escape the body's natural immune response [21, 22]. The importance of immune regulation in cancer progression can be explained by the increase in the number of immunosuppressive factors and cells and the lack of immune system-activating signals in the TME. TLRs are important receptors that activate immune cells and have been reported to play an important role in cancers, such as bladder cancer and colorectal cancer [23, 24]. This makes TLRs suitable targets for ligand drug discovery strategies to establish new therapeutics for cancer [25]. Hence, it is worthwhile to explore the role of TLRs in tumor development. TLRs can upregulate the expression of costimulatory molecules, such as CD40, CD80, and CD86, and cytokines, such as IL-12, thus stimulating other immune cells, including T lymphocytes [26, 27]. However, TLR expression can lead to tumor growth by stimulating other cells, including cancer cells [28].

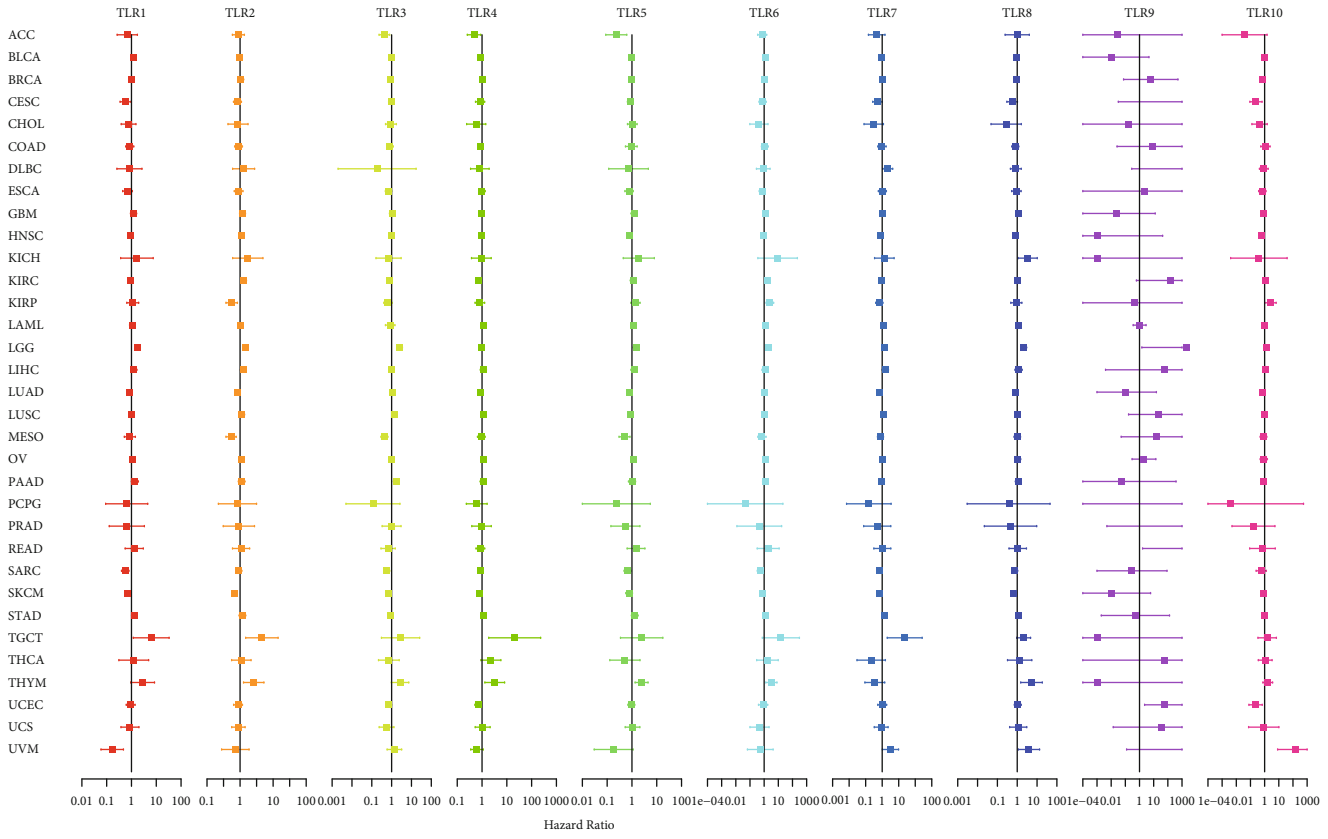
In this study, we explored the relationship between TLR transcriptional expression and TCGA tumor characteristics, including the TME, clinical significance, immune subtypes, stem cells, and drug response. We found that TLR isotypes have a significant effect on tumorigenesis. First, we analyzed the differential expression of 33 TCGA cancer types in 11,057 samples (including 10,327 tumor samples and 730 paracancerous samples). Through multidimensional analysis, we found significant differences in TLR expression levels among different cancer types. Survival and Cox proportional hazard regression analyses were also performed. For some types of cancers, we found a statistically significant difference in survival between patients with high and low TLR expression, suggesting that TLRs may be a potential prognostic indicator for clinical applications. Furthermore, we performed drug response analysis to explore the relationship between drug sensitivity and TLRs. This is expected to provide insights for new cancer therapies.

Table 2: Detailed information of the survival analysis of the TLRs family in pan-cancer with significant p value.

Gene	Cancer type	p value
TLR1	KIRC	0.034688969
TLR1	LGG	0.000296933
TLR1	SARC	0.026721888
TLR1	SKCM	0.000697683
TLR1	UVM	0.002732005
TLR2	LGG	1.65E-05
TLR2	LUAD	0.008433019
TLR2	MESO	0.017243009
TLR2	SKCM	2.17E-06
TLR2	TGCT	0.018921076
TLR2	THYM	0.009423895
TLR3	KIRC	2.94E-07
TLR3	KIRP	0.004130991
TLR3	LGG	0.000104245
TLR3	MESO	0.002692585
TLR3	PAAD	0.024365165
TLR3	SARC	0.009139017
TLR3	SKCM	0.000167396
TLR3	TGCT	0.042432124
TLR3	UCEC	0.031991718
TLR4	ACC	0.007177616
TLR4	KIRC	0.007164204
TLR4	LAML	0.044171562
TLR4	LUAD	0.028500171
TLR4	SKCM	7.46E-05
TLR4	TGCT	0.021867869
TLR4	THYM	0.020130819
TLR4	UCEC	0.00545563
TLR5	ACC	0.01419818
TLR5	ESCA	0.040248748
TLR5	LGG	0.014075805
TLR5	OV	0.036628212
TLR5	SKCM	0.022197327
TLR5	STAD	0.008021708
TLR5	THYM	0.005545537

Table 2: Continued.

Gene	Cancer type	p value
TLR6	BLCA	0.036456726
TLR6	ESCA	0.01912187
TLR6	KIRP	0.008085447
TLR6	LGG	0.003399361
TLR6	SKCM	0.003736075
TLR7	DLBC	0.032371891
TLR7	LAML	0.01921935
TLR7	LGG	0.00593611
TLR7	LUAD	0.000486804
TLR7	SARC	0.016297397
TLR7	SKCM	0.001124964
TLR7	UVM	0.03400155
TLR8	LAML	0.032090719
TLR8	LGG	0.003214408
TLR8	SKCM	1.26E-06
TLR8	THYM	0.020533224
TLR8	UVM	0.004248326
TLR9	KIRC	0.020215421
TLR9	LAML	0.038214202
TLR9	UCEC	2.97E-05
TLR10	CESC	0.02579765
TLR10	COAD	0.041270017
TLR10	HNSC	0.013737092
TLR10	LGG	0.003769908
TLR10	LUAD	0.000369848
TLR10	READ	0.028148853
TLR10	SARC	0.000659968
TLR10	SKCM	1.31E-05
TLR10	UCEC	0.003987076



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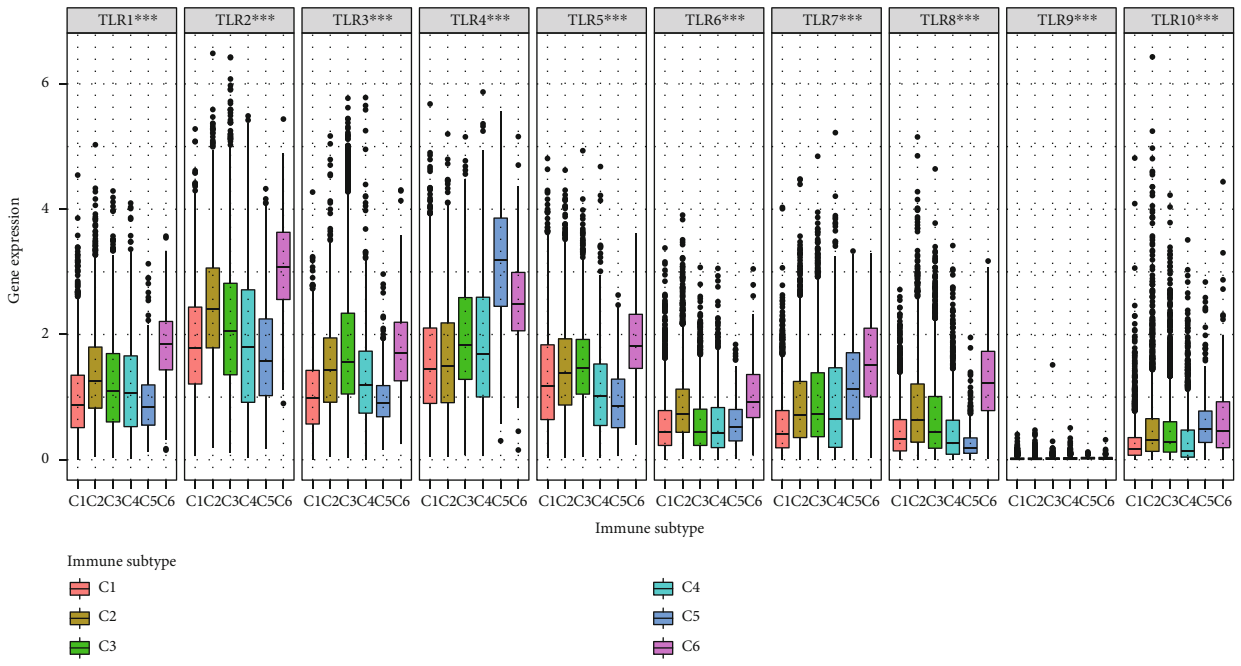

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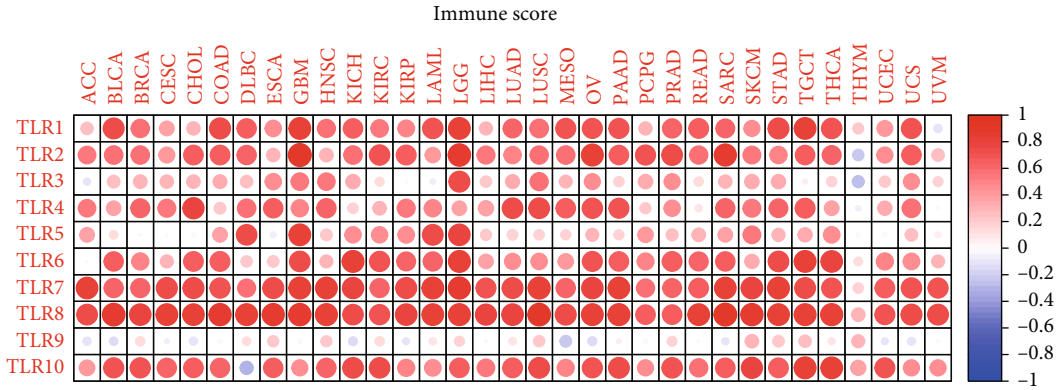
Figure 4: Cox regression and immune subtype analysis in pan-cancer. (a) Univariate Cox regression for each TLR gene in pan-cancer. (b) Differential expression of TLRs in differential immune subtype.



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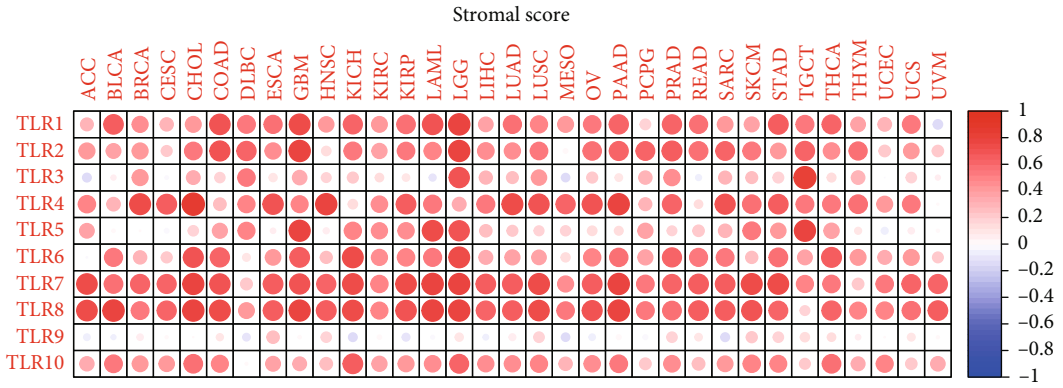

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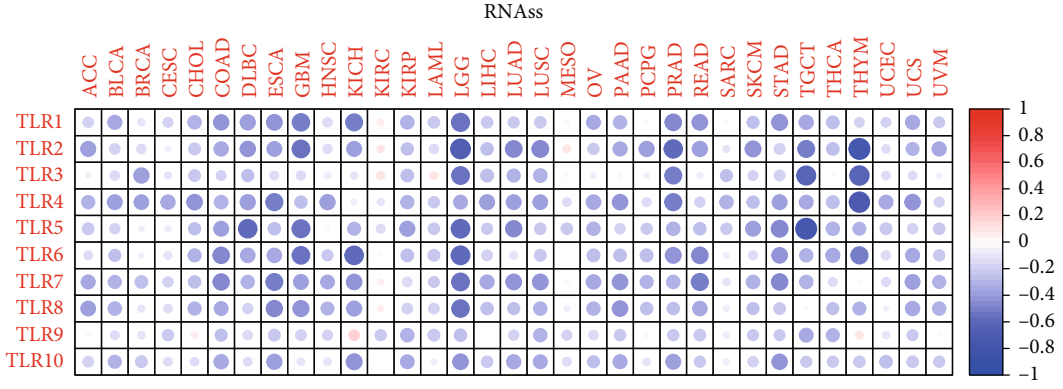

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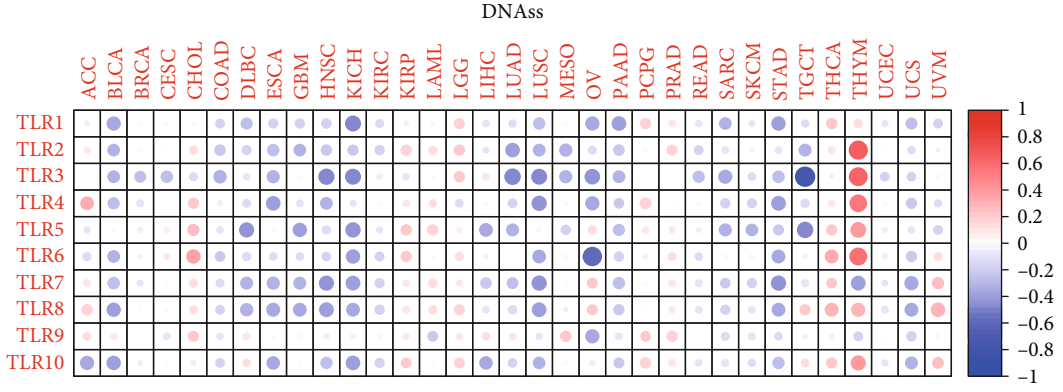

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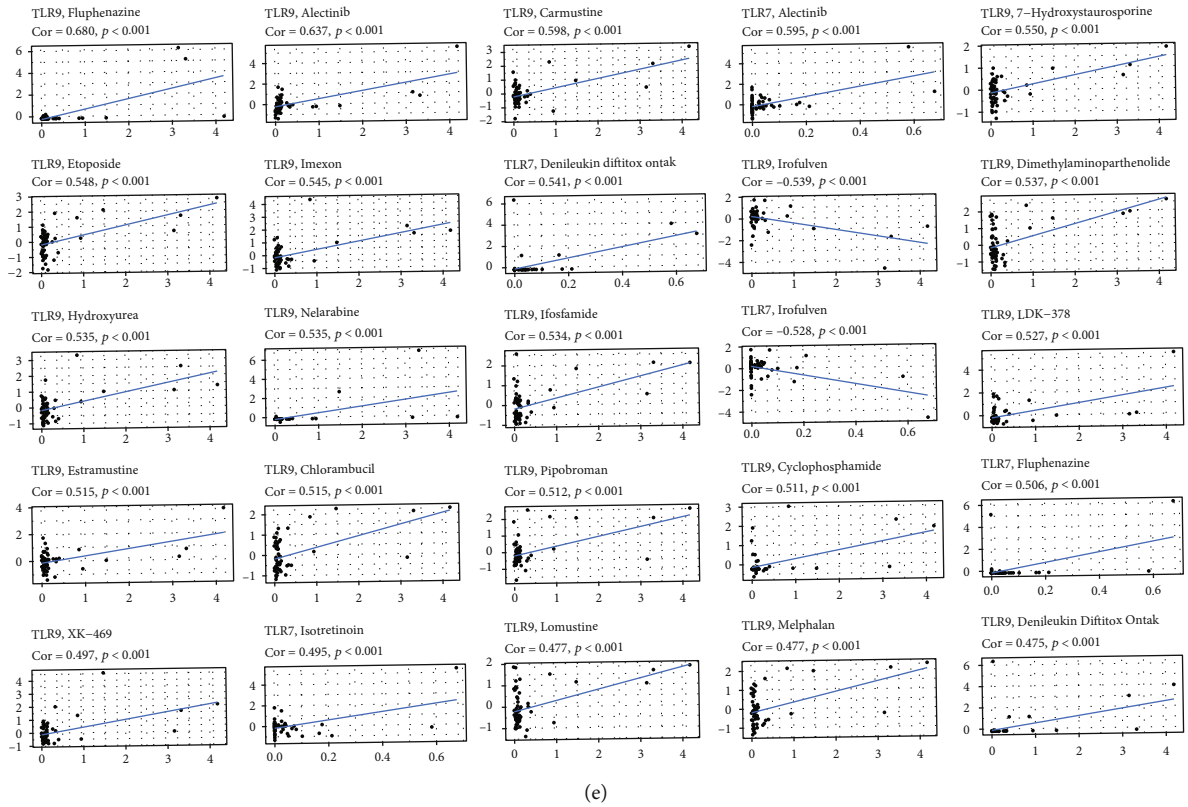


(e)

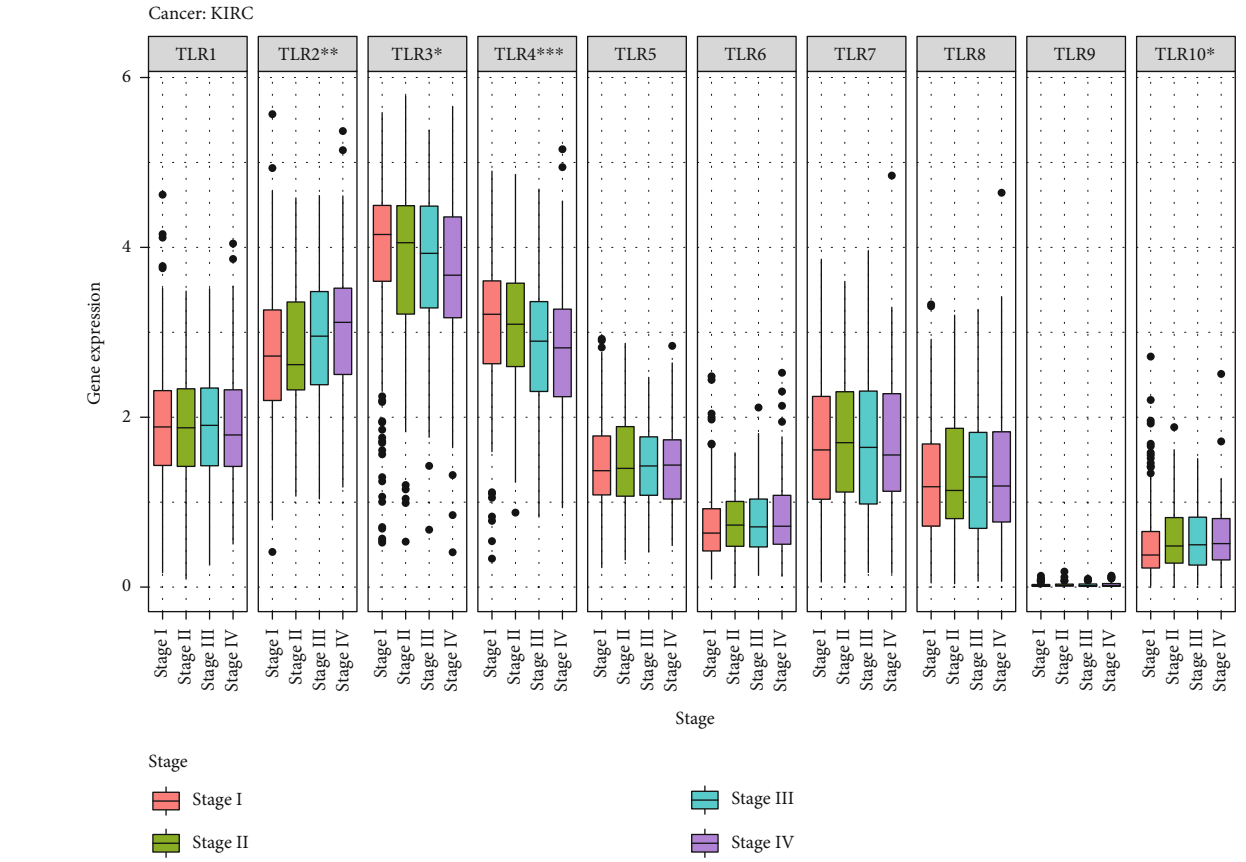
FIGURE 5: Stemness indices analysis, tumor microenvironment analysis, and drug sensitivity analysis in pan-cancer. (a) The correlation between immune score and expression of TLRs. (b) The correlation between stromal score and expression of TLRs. (c) The correlation between RNAss and expression of TLRs. (d) The correlation between DNAss and expression of TLRs. (e) Drug sensitivity of TLRs.

In our study, TLR2 was highly expressed in most cancer types. This result is similar to that of most previous studies [29–31]. Gergen et al. [32] reported that TLR2 activation induces the proliferation of lung adenocarcinoma cells by activating NF-κB. As a special link between lung cancer cells and mesenchymal stem cells in the TME, TLR2 promotes crosstalk and ultimately promotes changes in the tumor-supporting phenotype of mesenchymal cells [33]. Furthermore, the expression of TLR2 protein was shown to be upregulated in colon cancer and significantly correlated with a low overall survival rate of patients with colon cancer [34, 35]. Thus, the TLR2 signaling pathway may be an important potential therapeutic target in cancer.

In our study, we found that TLR9 was hardly expressed compared to the other TLR genes, which led to TLR9 showing less correlation with both immune and stromal scores. However, several studies have reported that TLR9 is associated with the development of cancers, especially gynecologic cancer [36, 37]. The activation of TLR9 on DCs and plasmacytoid DCs promotes the secretion of a large amount of type I IFN, which has both direct (tumor cell inhibitory effect) and indirect (antitumor immune responses) effects on cancer cells and is most evident in the early stages of antitumor immune responses [38].

Thorsson et al. [39] identified the immune landscape of cancer in the C1-C6 immune subtypes. In our study, we classified tumor samples by representative immune signatures and detected the RNA-seq levels of TLR 1-10 in C1 to C6. Interestingly, all TLRs were differentially expressed in different immune subtype samples. The TME, including the extracellular matrix, tumor vascular system, and tumor cell types, is closely related to immune functions and has an important impact on treatment response and clinical prognosis [40]. TLRs are expressed in the TME [41]. We further confirmed this information by extracting data on the fractions of stromal and immune cells in tumor samples from the 33 TCGA cancer types by calculating stromal scores, immune scores, and ESTIMATE scores. TLR expression was positively correlated with immune and stromal scores in almost all cancer types. On one hand, TLRs are expressed during programmed cell death induced by TME; on the other hand, they trigger the release of cytokines and chemokines in the TME and recruit immune cells to further release proinflammatory cytokines, angiogenic factors, and growth factors, such as TGF β, IL-8, CXCR4, ICAM-1, and VEGF. TLRs can repair the antitumor function and apoptotic response of antigen-presenting cells and effector T-cells [42, 43]. TLR signaling pathways play an essential role in controlling tumor progression, metastasis, recurrence, and chemotherapy tolerance through inappropriate immune enhancement and antitumor immunity [44].

Stemness was used to distinguish the stem cell-like characteristics of the tumor, such as self-renewal and dedifferentiation [45]. Two types of stemness indices were assessed:



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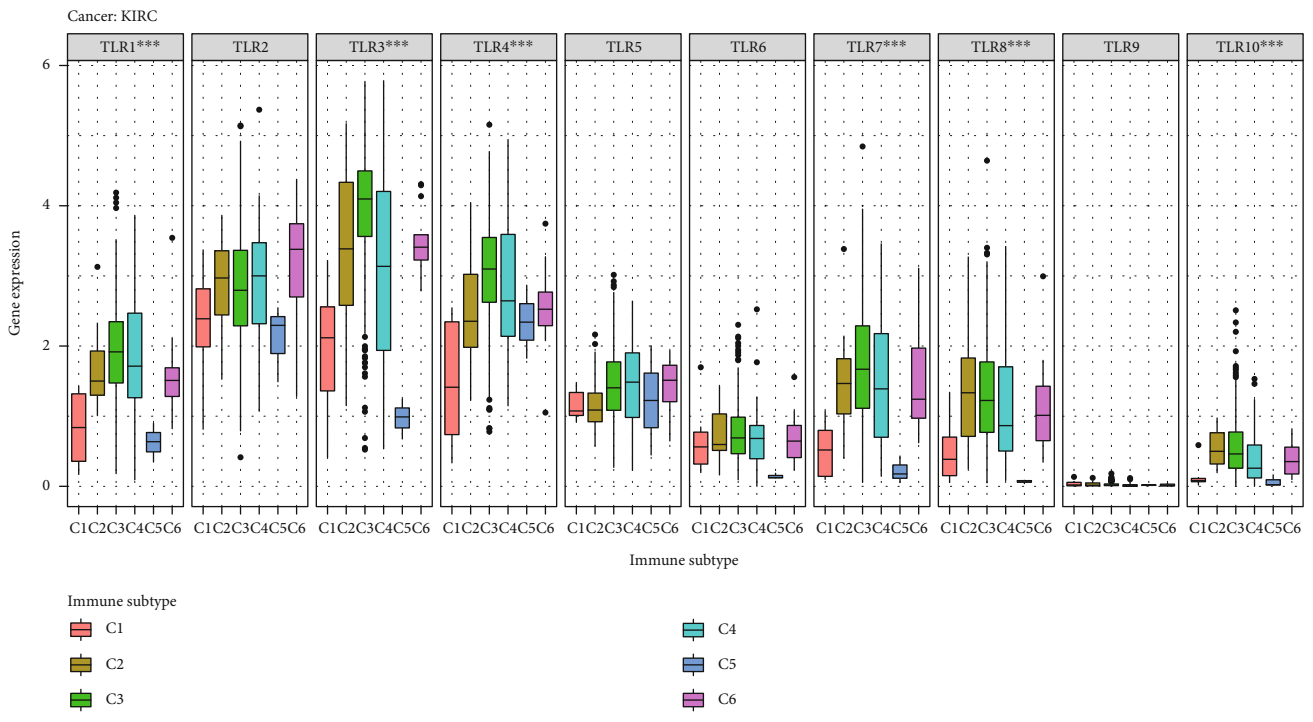

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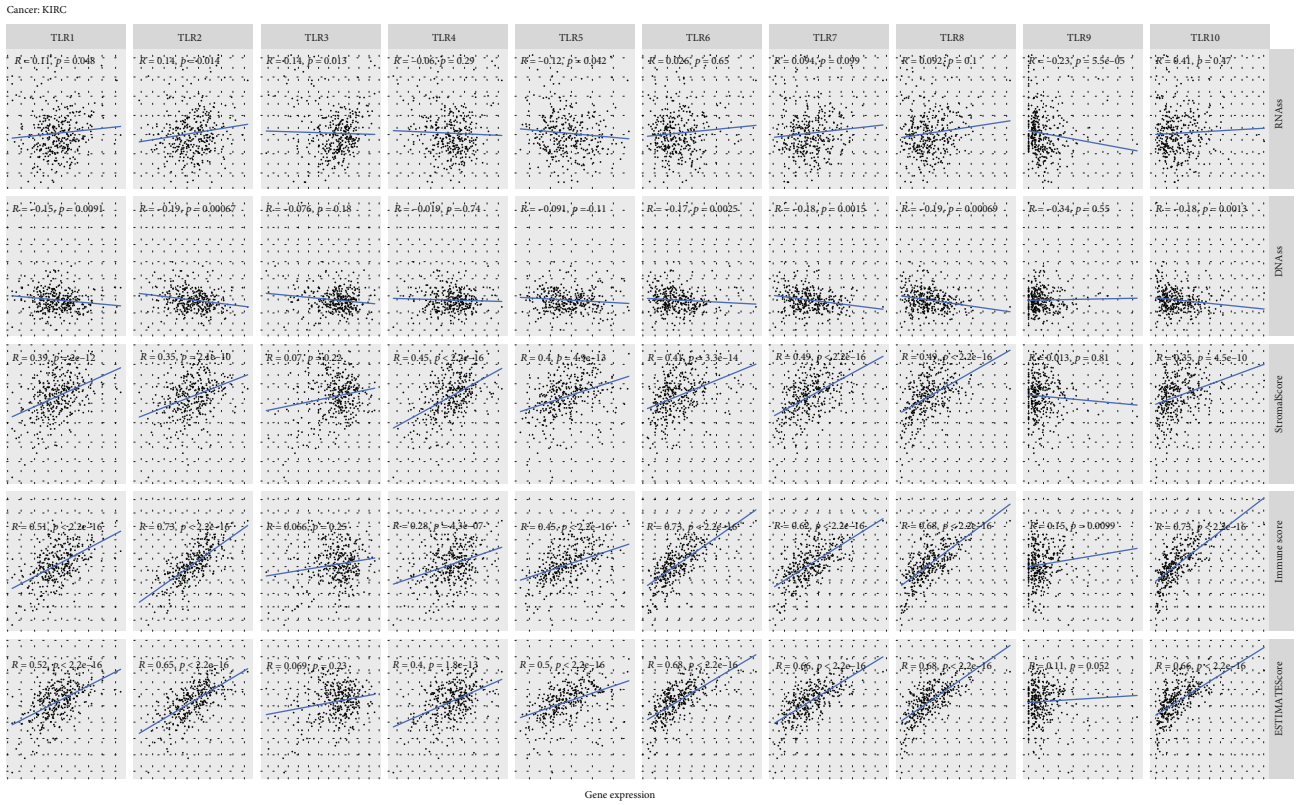

(c)

Figure 6: TLRs in KIRC. (a) Differential expression of TLRs between stage I and stage IV in KIRC. (b) Differential expression of TLRs in different immune subtype in KIRC. (c) Correlation between the expression of TLRs and stemness indices, tumor microenvironment.

DNAss and RNAss [46]. We found that the expression of TLRs was negatively correlated with RNAss in nearly all types of cancers, except KIRC, and negatively correlated with DNAss in many types of cancers, except ACC, CHOL, KIRC, LAML, LGG, TGCT, THCA, THYM, and UVM. TLR3 activation facilitates the expression of stemness-associated genes, including OCT3/4, NANOG, and SOX2 [47]. TLR4 expression in HCC is associated with increased stem-like properties [48]. NF-?B, activated by TLR signaling, was closely aligned with proliferation, invasion, and tumorigenesis [49].

Our study also found that the transcriptional expression levels of TLR7 and TLR9 were associated with drug response. Among them, the expression of TLR9 had a significant positive correlation with drug sensitivity to fluphenazine, alectinib, carmustine, and 7−hydroxystaurosporine. There was a significant positive correlation between TLR7 and the drug sensitivity of alectinib. These results have clinical relevance for guiding selection of antitumor therapies.

Finally, we explored the relationship between TLRs and KIRC. TLR2, TLR3, TLR4, and TLR10 were significantly differentially expressed between stages I and IV. TLR1, TLR3, TLR4, TLR7, TLR8, and TLR10 were significantly differentially expressed between C1 and C6 immune subtypes. All TLRs were positively correlated with immune, stromal, and ESTIMATE scores. Morikawa et al. [50] reported that TLR3 was overexpressed in KIRC, suggesting that the TLR3 pathway may be a novel therapeutic target in KIRC. Moreover, the expression of TLR9 is an independent prognostic marker of KIRC, and the loss of TLR9 expression is related to poor prognosis of KIRC [51]. Our results provide guidance for further exploration of the role of TLRs in KIRC.

Although this is the first study to multidimensionally analyze TLRs across multiple cancer types, it has some limitations. First, our results have not been verified using other independent databases; thus, it is necessary to validate the conclusions by generating our own data and using other public databases in the future. Second, this was a dry lab study [52], and we have not explored the underlying mechanisms behind the bioinformatics analyses through molecular and animal experiments. Finally, we studied the relationship between TLR family members and various combinatorial data. However, biometric correlations may not clarify the mechanisms of interaction and regulation directly; thus, further studies are needed to verify these potential mechanisms via laboratory-based molecular experiments. Further investigation is needed to determine the potential of TLRs and their coactivators as therapeutic targets in cancer.

5. Conclusions

TLRs were expressed differently in different cancer types and different immune subtype tissue and were positively

correlated with immune-estimate scores and stromal-estimate scores. The expression of TLR9 had a significant positive correlation with the drug sensitivities to fluphenazine, alectinib, carmustine, and 7−hydroxystaurosporine. TLR7 had a significant positive correlation with alectinib sensitivity. We demonstrated the significant pan-cancer role of the TLR family and potential therapeutic strategies for cancer. However, further laboratory studies are required to confirm our results.

Abbreviations

TLRs: Toll-like receptors
RNAss: mRNA expression-based stemness scores
KIRC: Kidney renal clear cell carcinoma
DNAss: DNA methylation-based stemness scores
ACC: Adrenocortical carcinoma
CHOL: Cholangiocarcinoma
LAML: Acute myeloid leukemia
LGG: Low-grade glioma
TGCT: Testicular germ cell tumors
THCA: Thyroid carcinoma
THYM: Thymoma
UVM: Uveal melanoma
PAMPs: Pathogen-associated molecular patterns
ssRNA: Single-stranded RNA
LPS: Lipopolysaccharide
DCs: Dendritic cells
TCGA: The Cancer Genome Atlas
HR: Hazard ration
OCLR: One-class logistic regression.

Data Availability

Source data of this study were derived from the public repositories, as indicated in the section of "Materials and Methods" of the manuscript. And all data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was not applicable for ethical approval, and source data of this study were derived from the public repositories.

Disclosure

A preprint has previously been published [53].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

PW and LC contributed to the design, analysis and interpretation of data, drafting of the manuscript, and critical revision of the manuscript; PW and HSY carried out the statistical analysis; PW, XY, HSY, and LC carried out the methodology; LC performed the project administration; PW and XY performed the writing (original draft); PW, HSY, XY, and LC performed the writing (review and editing).

Acknowledgments

We thank all the R programming package developer.

Supplementary Materials

Supplementary Table. Detailed information about the drug sensitivity of TLRs.13. *(Supplementary Materials)*

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