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Research Article
Therapeutic Impact of ODN2088 to Block TLR9 Activity in Induced Liver Fibrosis Mice

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Abstract

Background and Objective: Liver fibrosis is the result of an excessive accumulation of extracellular matrix that develops when inflammation and chronic injury form scar tissue in the liver. Toll-like receptor 9 (TLR9) plays a central role in the innate immune response by recognition of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). This study aimed to show the therapeutic effects of TLR9 antagonist oligonucleotide (ODN) 2088 on liver fibrogenesis. **Materials and Methods:** Mice were injected intraperitoneally with carbon tetrachloride (CCl_4) or corn oil twice weekly for up to 8 weeks. Mice were also injected with CpG ODN 2088 (50 µg/20 g) daily for the last 4 weeks. At sacrifice, the serum level of liver enzyme activity was measured. Expression of pro-inflammatory and pro-fibrotic biomarkers was analyzed in liver tissue. **Results:** TLR9 antagonist, CpG ODN 2088, remarkably decreased the haptic inflammation and fibrosis during CCl_4 administration. Treatment with CpG ODN 2088 resulted in reduced serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). That was paralleled with inhibition in the production of intrahepatic inflammatory and fibrotic factors including collagen, α-Smooth Muscle Actin (SMA), Transforming Growth Factor-beta (TGF-β), interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α). Proliferation (Ki-67) and apoptosis (caspase-3) markers were highly suppressed after CpG ODN 2088 administration. **Conclusion:** Our results indicate that TLR9 antagonist, ODN 2088, showed protective effects against hepatics inflammation and fibrosis in the CCl_4-induced fibrosis model. These observations suggest that ODN 2088 can be a potential therapeutic target for liver fibrosis treatment.

Key words: Liver fibrosis, inflammation, CCl_4, ODN 2088, TLR9, PAMPs, DAMPs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatic fibrosis is a mechanism linked to chronic injury caused by different processes. Worldwide, liver fibrosis is responsible for 1.4 million deaths each year[1,2]. Few therapeutic protocols are available for liver fibrosis treatment; therefore liver transplant remains the gold standard therapeutic procedure to treat the patient[3]. In industrial countries, the most prevalent risk factors for liver fibrosis progression are Non-Alcoholic Steatohepatitis (NASH), alcoholic steatohepatitis and Hepatitis C Virus (HCV)[1,4]. Hepatic Stellate Cells (HSCs) stimulation is counted as the main driver of liver fibrosis progression. During fibrosis, HSCs transdifferentiate to myofibroblasts and stimulate yielding of extracellular matrix (ECM) and α-Smooth Muscle Actin (α-SMA) proteins. Activation of HSCs is mainly regulated by factors produced by other liver cells like Transforming Growth Factor-beta (TGF-β), inflammatory cytokines, Platelet-Derived Growth Factor (PDGF) and chemokines. Hepatocyte death is an additional factor regulating the growth of liver fibrosis[5].

Toll-like receptors (TLRs) are a vast family of proteins that recognize various conserved components explicit by microbial pathogens, which are recognized as Pathogen-Associated Molecular Patterns (PAMPs)[6]. The participation of bacteria and bacterial PAMPs in liver fibrosis evolution has been reported[7]. Elevated bacterial translocation and circulating lipopolysaccharide (LPS) levels have been linked to advanced liver fibrosis[8]. Besides, a decrease in microbiota concentration was connected with a reduction in the degree of liver fibrosis and cirrhosis after the administration of non-absorbable antibiotics in rats[9]. TLR9 is highly expressed by HSCs during chronic liver injury. Further, TLR9 knockout mice developed reduced hepatic fibrosis after bile duct ligation in CDAA fed mice models of NASH[10]. It has been demonstrated that the expression of TLR9 by HSCs has an important function in the detection of DNA from apoptotic hepatocytes, which cause HSC activation[11].

Studies have shown that elevated amounts of TGF-β and collagen type I mRNA in HSCs can be detected by apoptotic hepatocyte DNA. Additionally, TLR9 and MyD88 can stamp down PDGF-induced HSC chemotaxis[10,12]. TLR9 can be triggered by CpG dinucleotides found in bacterial DNA, resulting in increased expression levels for the pro-fibrogenic chemokine monocyte chemotactic protein-1 (MCP-1). In addition, reduced hepatic fibrosis correlated with a lower concentration of hepatic MCP-1 and collagen expression detected in TLR9 null mice, compared with WT mice[11]. The study demonstrated the important role of TLR9, which is expressed in dendritic cells in liver fibrosis. In response to the binding of CpG-DNA with TLR9, dendritic cells from fibrotic liver released more TNF-α, IL-6 and cytokines, resulting in the promotion of liver fibrosis as a result of activation of HSCs and cytotoxicity of natural killer (NK) cells[13]. Chloroquine, a TLR9 inhibitor, acted as an anti-fibrogenic agent in mice injected with CCl_4 to induce fibrosis[11]. Chloroquine disturbed the HSC activation and autophagy pathways. It reduced the liver expression of α-SMA and TGF-β1 and the blood levels of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)[14]. Oligonucleotide ODN 2088 is a DNA sequence that inhibits the activity of TLR9 and its signaling pathways by disturbing the co-localization of unmethylated CpG dinucleotides with TLR9[15]. This study aimed to elucidate the effect of targeting TLR9 by ODN2088, on a mouse model for liver fibrosis.

MATERIALS AND METHODS

Study area: The study was carried out at the Regional Center for Mycology and Biotechnology, Azhar University, Egypt, from the 2nd of February to the 10th of April, 2019.

Ethics statement: All animal-based experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals under a protocol approved by the Local Committee of Bioethics, Jouf University, Sakaka, the kingdom of Saudi Arabia.

Reagents: The TLR9 antagonist CpG ODN2088 (5'-tcctggcggggaagt-3') was obtained from Invitrogen (CA, USA). CCl_4 and corn oil were bought from Sigma-Aldrich (St. Louis, MO, USA).

Animal experiments: Total 24, 4-6-week-old male BALB/c mice were domiciliated in standard polypropylene crates under insured environmental conditions with maintained light-dark cycles (12 hRS light and 12 hrs dark. Sacrifice of animals was done under local anesthesia using ether. Intraperitoneal (IP) injection of 5 mL kg^{-1} of CCl_4 solution (CCl_4 diluted in corn oil, 1:4) for 8 weeks was used to induce fibrosis in the mice.

Experimental design: Twenty-four BALB/c male mice were placed into four groups (n = 6 for each) as follows:

Group 1: Vehicle that received only corn oil (5 mL kg^{-1} intraperitoneally) two times each week for two months

Group 2: Fibrosis-induced mice injected with CCl_4 (5 mL kg^{-1} intraperitoneally) two times each week for two months

Group 3: Vehicle mice that received corn oil for 8 weeks and injected with CpG ODN 2088 (50 µg/20 g b.wt., re-suspended in endotoxin-free water) twice a week for the last 4 weeks

Group 4: Fibrosis-induced mice that treated with CCl_4 for 8 weeks and injected with CpG ODN 2088 (50 µg/20 g body weight re-suspended in endotoxin-free water) once a day for the last 4 weeks

About 48 hrs after the last injections, mice were weighed, euthanized and sacrificed. A post-mortem examination was carried out to detect abnormal changes in the livers. Livers were harvested and weighted. Blood samples were collected and centrifuge at 1000×g for 15 min to obtain serum. A small piece of hepatic tissue was fixed using 10% neutral buffered formalin and the rest of the hepatic tissue was kept at -80°C for further experiments.

Histopathological evaluation: Liver tissues were processed using a standard histopathological protocol to evaluate the morphological and pathological changes with H and E staining. Liver fibrosis severity was assessed by Masson's trichrome staining technique[16].

Immunohistochemistry: Changes in protein expression were evaluated using 5 µm thick sections cut from formalin-fixed, paraffin-embedded tissue. Liver sections were de-paraffinized in xylene followed by a rehydration step using ethanol. The tissue sections were firstly treated with 3% hydrogen peroxide for 4 min to block the endogenic peroxidase; this was followed by 10 min in 10% BSA to block non-specific protein binding sites. Slides put in an overnight incubation with primary antibodies as follows: anti-SMA (Abcam) 1:1000 dilution; anti-TGF-β (Abcam) 1:500 dilution, anti- cleaved caspase (Abcam) 1:100 dilution; anti-Ki-67 (Abcam) 1:100 dilution; IL-6 antibody (Abcam) 1:100 dilution; and anti-TNF alpha antibody (Abcam) 1:100 dilution. The next day, PBS washes for 5 min were applied three times. Then, incubation with the relevant secondary antibody (Abcam) diluted in PBS, for 1 hr at 25°C, followed by three PBS washes. Then, sections were incubated with diaminobenzidine tetrahydrochloride solution for 10 min. Counterstain is done after washing slides with distilled water. Morphometric data were obtained from examination of these sections using an Olympus DP2-BSW Image computer system (Olympus Imaging System, Tokyo, Japan) and the ImageJ software program (version 4.5.1.27). Five non-overlapping fields on slides were used to calculate the mean of the percentage area of α-SMA, TGF-β, TNF-α, Ki-67, caspase-3 and IL-6 reactions at 400X magnification.

Liver enzyme activity measurement: AST and ALT activity measured by clinical chemistry analyzer according to the manufacturer's protocol (Beckman Counter LX20, USA).

Statistical analysis: Data and results analysis was done through one-way ANOVA and Tukey's multiple group comparison tests using Graph Pad Prism software for windows version 8.0.0 (Graph Pad, San Diego, CA, USA, www.graphPad.com). All results are expressed as mean± standard error of the mean (SEM). To consider a p-value statistically significant it should be less than 0.05.

RESULTS

Liver weight and liver weight-to-body weight ratio: Liver weight and liver weight-to-body weight ratio for mice injected with CCl_4 showed non-significant changes in comparison with the vehicle as well as vehicle mice that received ODN 2088. Further, liver weight or liver weight-to-body weight ratio showed no significant differences between mice injected with CCl_4 alone or those injected with CCl_4 and co-treated with ODN 2088 (Table 1).

Histopathological evaluation of liver damage: Microscopic evaluation of liver sections from both the vehicle mice revealed normal cytoarchitecture of the hepatic parenchyma with distinct strands of hepatocytes around central veins (Fig. 1a). Thickening of hepatic capsules due to fibrous tissue proliferation with the extension of fibrous strands from these capsules into the neighboring hepatic lobules was noticed in CCl_4 treated mice. Mononuclear cellular infiltration of the

Table 1: Mean of body weight (g), liver weight (g) and liver weight (g)/body weight (g) ratio in study groups

Parameters	Vehicle	CCl_4	Vehicle+ODN2088	CCl_4+ODN2088
Body weight (g)	37.2±3.07	32.67±3.64	34.85±3.41	33.00±1.41
Liver weight (g)	2.6±0.54	3.34±0.31	3.08±0.37	3.32±0.60
Liver/body weight ratio	12.6±1.43	9.80±0.79	11.23±1.23	10.09±1.39

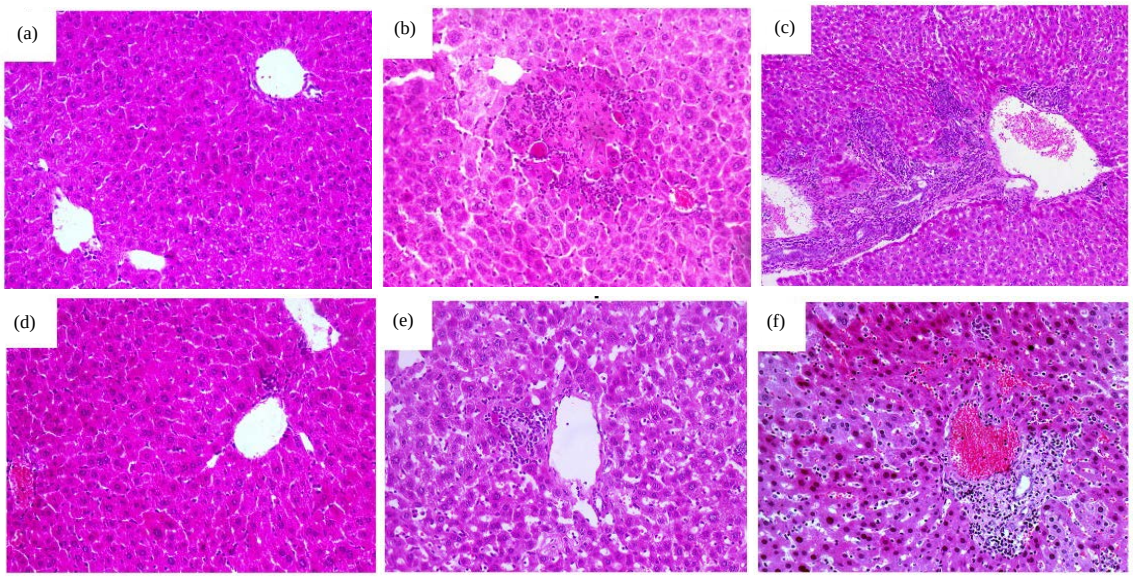

Fig. 1(a-f): Histological differences in mice liver after CCl_4 and ODN 2088 treatment, (a) Vehicle, (b) CCl_4, (c) CCl_4, (d) Vehicle+ODN 2088, (e) CCl_4+ODN 2088 and (f) CCl_4+ODN 2088

portal areas, particularly of lymphocytes was also detected. The majority of the live sections examined showed severe hepatic steatosis and/or diffuse hydropic degeneration of hepatocytes. Moreover, focal mononuclear cellular hepatic parenchyma infiltration was reported beside the existence of multiple zones of hepatic necrosis (Fig. 1b). Conversely, livers examined from CCl_4-treated mice showed pronounced portal fibrosis with portal- portal and portal-central bridges (Fig. 1c). Moreover, microscopic examination of liver sections from vehicle with ODN 2088 mice also showed normal cytoarchitecture of the hepatic parenchyma with distinct strands of hepatocytes around central veins same as the vehicle group (Fig.1d). In addition, pyknosis of the nuclei of some hepatic cells in the absence of hepatic fibrotic lesions was recorded in some of the livers examined from this group (Fig. 1e). The livers examined from CCl_4 plus ODN 2088 mice showed hepatic fibrotic lesion regression relative to the CCl_4-treated group, where mild peribiliary fibrosis and lymphocytic cellular aggregation were seen (Fig. 1f).

Oligonucleotide 2088 prevents liver fibrosis: To evaluate the protective effects of ODN 2088 on hepatic fibrogenesis induced by CCl_4, Masson's trichrome-stained liver tissue sections were used to test for the deposition of collagens. Microscopic examination of livers from group 1 mice revealed only pericellular and perivascular thin strands of green-stained collagen (Fig. 2a). Conversely, the CCl_4-treated group showed significantly more collagen fiber deposition in which green collagen staining was prevalent in most portal areas of the examined liver-with the establishment of dense fibrotic partitions between the hepatic lobules and the formation of portal-portal and portal-central bridges (Fig. 2b). Pericellular and perivascular thin strands of green-stained collagen also shown in group 3 liver sections (Fig. 2c). Regarding the microscopic examination, of the ODN 2088+CCl_4 group liver sections there was significantly less collagen fibrous deposition and green collagen staining was restricted to the portal area, particularly around the bile ducts (Fig. 2d). Furthermore, α-SMA expression was assessed in vehicle, most portal and central veins showed immunologically positive cells that present only in the walls; while other cells remained immunologically negative (Fig. 2e). In contrast, liver sections of CCl_4-treated mice showed significantly higher positive area percentages and had more deeply stained α-SMA-positive HSCs distributed majorly between the hepatic lobules, particularly in fibrotic septa bridging and portal areas when compared to vehicle mice (Fig. 2f). Vehicle-injected with ODN 2088 also showed immunologically positive cells that present only in the walls same as a vehicle (Fig. 2g). However, in CCl_4-induced hepatic fibrosis mice treated with ODN 2088, the α-SMA positive area percentage was significantly lower and less deeply stained α-SMA-positive HSCs were restricted to the portal areas compared to vehicle mice (Fig. 2h). The examined liver of vehicle mice showed weak expression of TGF-β (Fig. 2i), while the CCl_4-treated group showed a significantly higher illustration of TGF-β, along with the presence of numerous TGF-β-positive cells, particularly in the centrilobular areas (Fig. 2j). Vehicle mice treated with ODN

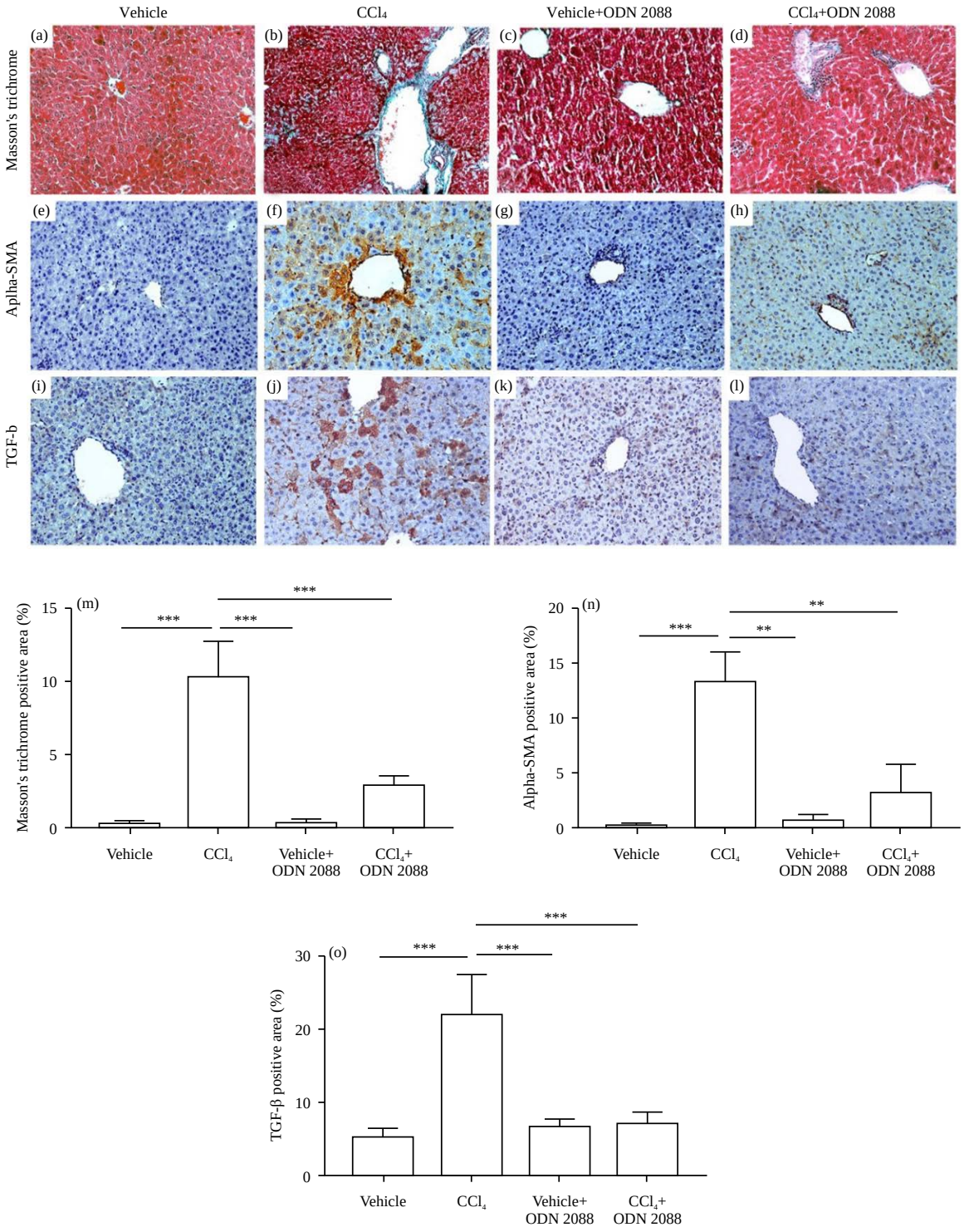

Fig. 2(a-o): Fibrotic differences in liver sections associated with treatment by CCl_4 and ODN 2088

2280 showed weak expression of TGF-β (Fig. 2k). Administration of ODN 2088 to CCl_4-induced fibrosis mice was related with a significant decrease in the percentage area of TGF-β immunoreactivity and a reduction in the number of TGF-β-positive cells (Fig. 2l). Treatments with CCl_4 result in a significant increase of positive area percentage detected by Masson's trichrome (Fig. 2m), α-SMA-positive area percentage (Fig. 2n) and TGF-β-positive area percentage (Fig. 2o).

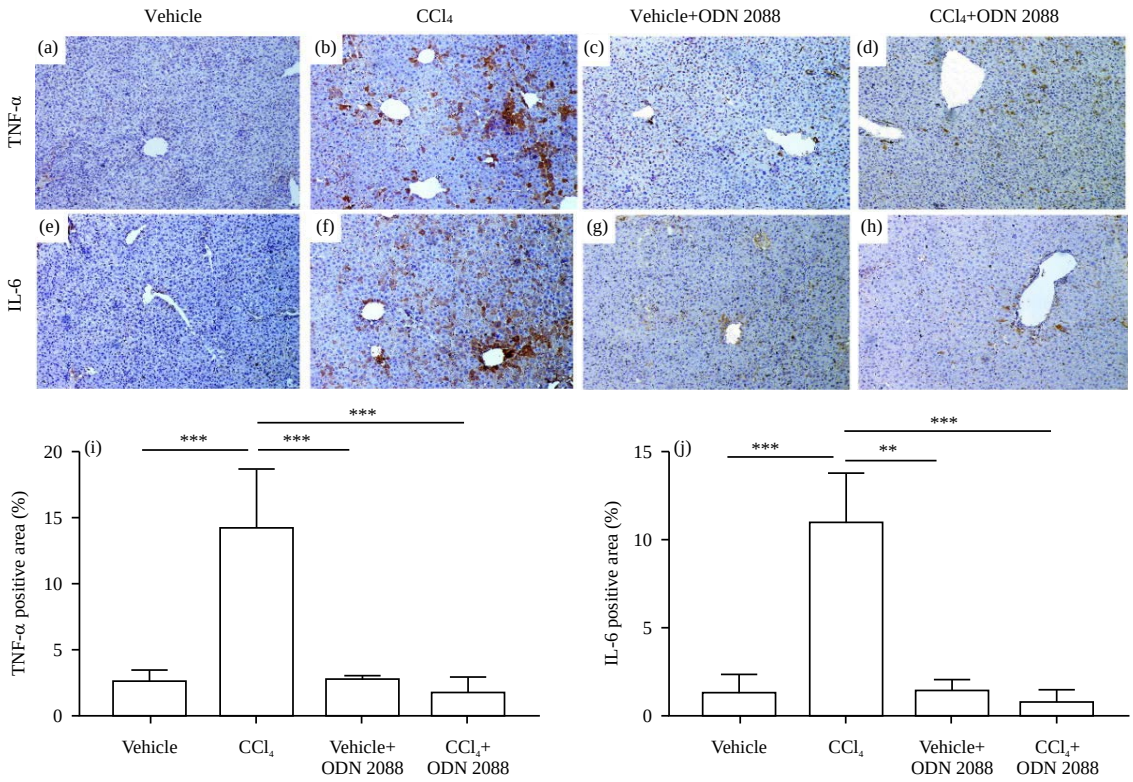

Fig. 3(a-j): ODN 2088 modulates the expression of pro-inflammatory cytokines in hepatic tissues

Oligonucleotide 2088 modulates the expression of liver pro-inflammatory cytokines: Weak immunoreactivity of TNF-α was detected in the vehicle (Fig. 3a). CCl_4-treated group showed significantly higher positive area percentages of TNF-α (Fig. 3b). Vehicle treated with ODN 2088 only showed weak immunoreactivity of TNF-α that limited for the sinusoidal cells with sparse positive cells (Fig. 3c). In addition, numerous cells positive for TNFα were recorded throughout the hepatic tissues therefore, administration of ODN 2088 for CCl_4 treated group was associated with smaller numbers of TNF-α- stained cells (Fig. 3d). Vehicle cells showed the same weak immunoreactivity for IL-6 (Fig. 3e), while the CCl_4 treated group showed a significantly higher positive percentage stained area for IL-6 (Fig. 3f). Vehicle-treated with ODN 2088 mice group showed weak immunoreactivity for IL-6 (Fig. 3g). Administration of ODN 2088 for CCl_4 treated group was associated with smaller numbers of IL-6 stained cells (Fig. 3h). CCl_4 administration lead to a significantly higher percentage areas of both TNF-α and IL-6 (Fig. 3i-j).

Oligonucleotide 2088 decreases the expression of cellular markers for proliferation and apoptosis: Liver sections of CCl_4-induced fibrosis mice exhibited significantly higher counts of Ki-67 positive cells compared with vehicle mice (Fig. 4a-b). In addition, a limited count of Ki-67-positive cells was noticed in liver section of the vehicle treated with ODN 2088 only and CCl_4+ODN2088 groups (Fig. 4c-d). Liver sections from vehicle group showed weak expression of nuclear and cytoplasmic caspase-3, most of which was located on blood vessels walls and sinusoids (Fig. 4e). Conversely, markedly diffuse cytoplasmic and nuclear caspase-3 expression with considerable caspase-3-positive cells were seen in examined CCl_4-induced fibrosis mice livers, especially around central veins (Fig. 4f) in compare to vehicle treated with ODN2088 group that showed weak expression of nuclear and cytoplasmic caspase-3 (Fig. 4g). CCl_4-induced fibrosis mice treated with ODN 2088 had much lower expression of hepatic caspase-3 (Fig. 4h). Treatment with ODN 2088 only was associated with substantially fewer Ki-67-positive cells than the CCl_4-induced fibrosis model group (Fig. 4i) and a significantly smaller percentage area of caspase-3 immunoreactivity (Fig. 4j).

Oligonucleotide 2088 restores liver enzyme activity in CCl_4-induced fibrosis mice: IP injection of CCl_4 was associated with significantly higher levels in serum of ALT and AST than was observed in control group mice. Mice treated with ODN 2088 had serum ALT and AST levels very similar to those in the control group (Fig. 5a-b).

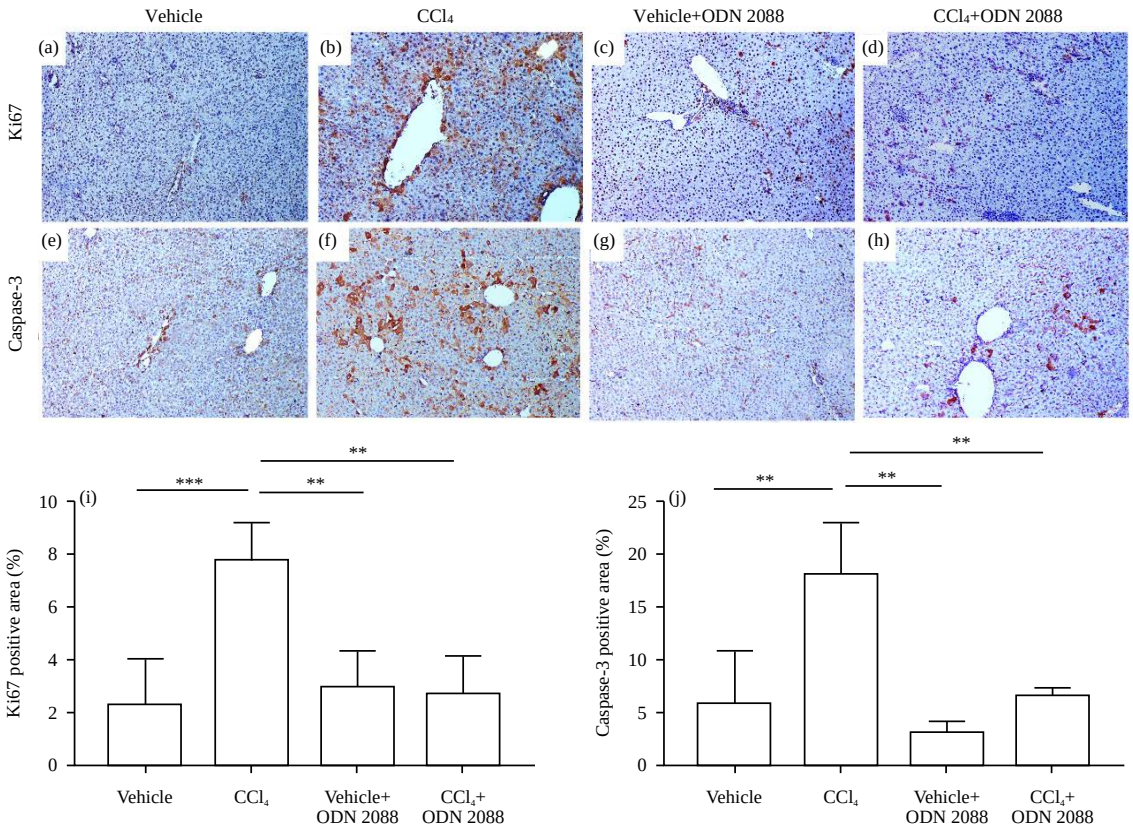

Fig. 4: ODN 2088 reduces the expression of cellular markers for proliferation and apoptosis

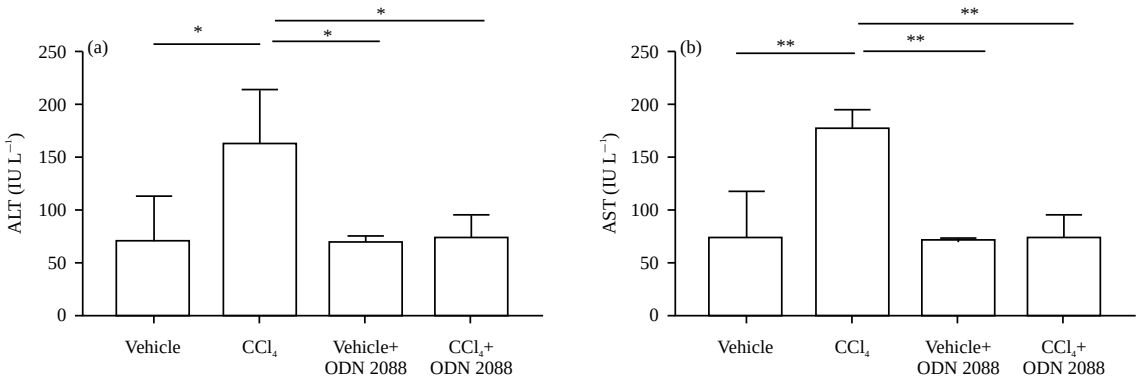

Fig. 5: ODN 2088 restores liver enzyme activity in CCl_4-induced fibrosis mice

DISCUSSION

This study has investigated whether ODN2088 plays a role in modulating fibrosis in CCl_4 induced-fibrosis mice. Liver fibrosis is a wound-healing reaction results from chronic hepatic damage due to several pathological processes mainly chronic viral hepatitis, cholestasis and autoimmunity. Deposition of excess fats in hepatocytes leads to a condition known as non-alcoholic fatty liver disease (NAFLD)[17]. When a patient's liver is compromised by varying degrees of cell death, inflammation and collagen deposition, NAFLD can progress to NASH[18]. NASH can eventually lead to extreme fibrosis and cirrhotic end-stage liver disease, potentially evolving into hepatocellular cancer (HCC)[19]. Hepatic cirrhosis is popular in end-stage liver fibrosis and is usually connected with severe complications like portal hypertension, hepatic failure and HCC[20].

As the first line of defense against invading pathogens, TLRs are commonly found in both immune and non-immune cells and suppressed in normal hepatocytes[21]. Recent studies have marked out the function of TLRs, ligands and intracellular signaling in liver fibrosis pathology and the possible roles of TLR signaling in hepatic fibrosis that linked to various hepatic disorders[6]. During progression to fibrosis, all hepatic cells undergo specific changes including capillarization of the sinusoids, Kupffer cells are activated to produce chemokines and cytokines and, finally, quiescent stellate cells are stimulated to produce ECM proteins like collagen 1[22]. Activation of HSCs contributes to the loss of their retinoid, which promotes the expression of new markers like PDGF, TGF-β and α-SMA. These factors are responsible for the proliferation of activated HSCs and the synthesis of ECM proteins to produce the fibrous scar[22]. Kim *et al.*[23] and Fujii *et al.*[24] illustrated that hepatic tissue from CCl_4-induced fibrosis mice had some cellular damage and centrilobular congestion without inflammatory cell infiltration. Along with the elevation of serum AST and ALT levels by CCl_4 treatment, this indicates liver injury. Moreover, severe fatty changes and early signs of fibrosis were observed. In our study, administration of ODN 2088, which acts on hepatocytes and stellate cells, was associated with markedly lower α-SMA expression, which acts as a characteristic feature of activated HSCs and is considered a hepatic fibrosis predictor. Our results also demonstrated markedly lower percentage areas of TGF-β immunoreactivity, smaller numbers of TGF-β-positive cells and smaller hepatic fibrotic lesions with significantly lower collagen fibrous sedimentation in the liver of mice treated by CCl_4 co-administered with ODN 2088. There was no difference in liver weight or liver weight-to-body weight ratio. Liver enzymes also occurred at nearly normal levels. These findings clearly show the ability of ODN 2088 to decrease collagen, α-SMA and TGF-β synthesis, which could be attributed to its anti-proliferative effect on HSCs.

The decrease in hepatocyte oxygenation, nutrients, or any cause of irritation results in activation of DAMPs that modulates pattern recognition receptors (PRRs) for liver immune cells to liberate inflammatory mediators, leading to increased late-stage hepatocyte death, which may result in liver failure[25,26]. This occurs through the activation of TLRs that results in the initiation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-associated protein kinase (MAPK), resulting in pro-inflammatory genes defining[27]. TNF-α can activate different signaling pathways involving inflammation, proliferation and apoptosis. Moreover, TNFα had a critical action in the pathogenesis of chronic inflammation of the liver that promotes liver fibrosis[28]. IL-6 had a crucial act in the primary control of acute and chronic inflammation. In chronic inflammatory diseases, serves as a pro-inflammatory cytokine, while in an acute inflammation it shows anti-inflammatory effects. Therefore, IL-6 is used as a classic pro-inflammatory cytokine biomarker in chronic liver fibrosis[29]. In our study, quantitative measurement and immunohistochemical evaluation for the presence of TNF-α and IL-6 was carried out to evaluate the anti-inflammatory potential of ODN 2088. CCl_4-induced fibrosis mice after administration of ODN 2088 showed a very low number and area percentage of the immunoreactivity of TNF-α- and IL-6-positive cells than in control mice. This finding indicates that ODN 2088 has a powerful anti-inflammatory effect, which may be attributable to its ability to block pro-inflammatory signaling pathways stimulated by TLR9.

Watanabe *et al.*[10] reported that DNA from mammalian apoptotic cells stimulated immune cells via TLR9. Use of TLR9 antagonists resulted in the downregulation of HSC differentiation. DNA mediated through TLR9, as well as apoptotic hepatocyte DNA, increased matrix precipitation and caused morphological modifications linked with HSC stimulation through TLR9. Further confirming this apoptosis-dependent hypothesis is the proportional ability of DNA from normal hepatocytes to cause such changes. In addition, an increase in expression of the common antigenic protein Ki-67 is used as a proliferation marker. Administration of ODN 2088 to CCl_4-induced hepatic fibrosis in our study associated with a strong reduction in hepatic caspase-3 expression. These results indicate that ODN 2088 reduces hepatic apoptosis and prevents HSC stimulation and liver fibrosis, which is induced by hepatocytes apoptotic response. Therefore, these findings suggest that ODN 2088 as a TLR9 antagonist could be a useful immune modulator to prevent apoptotic hepatocyte DNA and act as a significant mediator in liver fibrosis via lowering the population of Ki-67-positive cells.

CONCLUSION

This study concludes that ODN 2088 has immense anti-fibrotic potential to inhibit the progression of liver inflammation and fibrosis in mice models of hepatic fibrosis.

Administration of ODN 2088 led to reduced α-SMA, TGF-β and collagen production in the liver of mice treated with CCl_4. Moreover, serum liver enzymes were at nearly normal levels. Additionally, ODN 2088 significantly attenuated the hepatic expression of pro-inflammatory cytokines, TNF-α and IL-6.

SIGNIFICANCE STATEMENT

This study discovers the possible therapeutic effect of ODN2088 that can be beneficial for CCl_4-induced fibrosis rats. This study will help the researcher to uncover the critical area of gene therapy that many researchers were not able to explore. Thus, a new theory on this designed oligonucleotide and possibly other combinations, may be arrived at.

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