

Contribution of Outer Membrane Porin (Omp) K36 to *Klebsiella pneumoniae* induced Liver Abscess

Junn-Liang Chang^{1,2}, Wen-Cheng Tsai³ and Jiun-Han Chen^{4*}

¹Department of Pathology and Laboratory Medicine, Taoyuan Armed Forces General Hospital, Taoyuan City, Taiwan,

²Biomedical Engineering Department, Ming Chuan University, Taoyuan City, Taiwan

³Department of Medicine, Zuoying Branch of Kaohsiung Armed Forces General Hospital, Kaohsiung City, Taiwan

⁴Department of Medical Laboratory Science and Biotechnology, Yuanpei University of Medical Technology, Hsinchu, Taiwan

Abstract

Introduction: Pyogenic liver abscess is usually caused by multiple bacterial infections. This disease can be caused by single microorganism, *Klebsiella pneumoniae* that has been reported in Asia, North America and Europe. Many virulence factors of *K. pneumoniae* have identified including capsular polysaccharide, lipopolysaccharide, serum resistance, adhesins and siderophores. Previously, we demonstrated the virulent effect of outer membrane porin (OMP) K 36 by a lethality study. However, the role of OmpK36 in liver abscess pathogenesis is still unclear.

Objective: In this study, a virulent *K. pneumoniae* strain NVT-1002 and OmpK36 deficient mutant (Δ OMPK) were used to examine the contribution of OmpK36 to liver abscess. Liver injuries and inflammatory cytokines expression were detected by tissue section and enzyme linked-immunosorbent assay respectively. The toxic effect of OmpK36 to human hepatoma cell (HepG2) was tested by bacterial infections and the treatment with OmpK36 recombinant protein.

Results: The Results demonstrated that the NVT-1002 induced sever liver injuries while little or no damage was found in mice injected with OmpK36. Expression of serum and liver cytokines including TNF- α , IL-1 β , IL-6 and IL-10 were elevated after injection of NVT-1002, meanwhile, only IL-1 β was transiently elevated in OmpK36 group. Interestingly, OmpK36 recombinant protein showed no toxic effect to HepG2 cells, and the virulence of OmpK36 mutant to HepG2 was not altered compared to NVT-1002 group.

Conclusion: In conclusion, OmpK36 contributes to the *K. pneumoniae* induced liver abscess and inflammatory responses. The virulent effect of OmpK36 is not mediated by the protein itself, but other uncertain mechanisms. OmpK36 may be a therapeutic target in *K. pneumoniae* infection.

Introduction

Klebsiella pneumoniae have been documented as the common factor for cryptogenic liver abscess in Asia Pacific [1,2]. Similar findings in North America and Europe have also been reported [3-5]. Several virulent factors of *K. pneumoniae* were discovered and these factors are almost related to specific capsular polysaccharides (CPS) [2,6-8]. Accumulating reports have indicated that the capsule is essential to the virulence of *Klebsiella* species [9-11]. Up to now, the capsular antigen have been classified into 77 serotypes, and serotype K1 and K2 capsular antigen were found to comprise virulence in mouse peritonitis model, whereas isolates of serotypes other than K1 or K2 were with little or no virulence [5,12].

Outer membrane porins (OMP) are proteins that cross a cellular membrane and contribute to the diffusion of molecules. Many studies have showed OMPs play important roles in antimicrobial resistance [13, 14]. Previously, we have demonstrated the virulent effects of OMPK 36 by an animal study [15]. In this report, the results of animal lethality study showed that the virulence of OmpK36 mutant was reduced about 100 fold compared to the wild-type strain NVT-1002, which was isolated from a patient with liver abscess. However, the participation of OmpK36 in *K. pneumoniae* induced liver abscess formation and the toxicity to cells are still unclear. In this study, we examined the role of OmpK36 in *K. pneumoniae* induced pathogenesis by animal model and the inflammatory cytokines were also detected. In addition, the direct toxic effects of OmpK36 and whole bacteria were detected by the cell model.

Materials and Methods

Mice liver abscess study

Klebsiella pneumoniae isolates NVT-1002 and Δ OmpK36 were obtained as previously [15]. The adult BALB/c mice were injected intraperitoneally with 103 cfu of *K. pneumoniae* NVT-1002 or Δ OmpK36-NVT-1002 in 0.1 mL phosphate buffered saline. Mice were sacrificed with CO₂ after 1-3 days bacterial injections, the livers were removed, fixed with paraformaldehyde and embedded in paraffin. Histological sections were observed after hematoxylin-eosin (H&E) staining.

Kinetic of inflammatory cytokines production in liver and serum

Liver was removed for tissue section and supernatant of each sample was collected for cytokines production. Cardiac blood samples were collected under aseptic conditions. Blood samples were allowed to clot at 40°C and then centrifuged at 15000 rpm for 3 min. Serum samples

Corresponding Author: Prof. Jiun-Han Chen, Department of Medical Laboratory Science and Biotechnology, Yuanpei University of Medical Technology, Hsinchu, Taiwan, 306, Yuanpei Street, Hsinchu, Taiwan; E-mail: jhchen@mail.ypu.edu.tw

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were preserved at -80°C until measurement of the cytokine. Cytokine concentrations in liver and serum were measured with a mice cytokine 10-plex antibody bead kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Tumor necrosis factor (TNF)- α , interleukin (IL)- 1β , IL-6 and IL-10 levels were determined. The supernatants from homogenized liver were then withdrawn at the indicated times, and dilutions were assayed in triplicate for each independent experiment. All samples were tested in triplicate. The minimal detectable protein concentration was 10 pg/ml.

Construction of pET30a-OmpK36

The DNA fragment of OmpK36 was amplified by primer pairs OmpK36F (5'-GGGAATTCATATGCACCATCATCATCATCATA TGAAAGTTAAAGTACTG-3') and OmpK36R (5'-CCGCTCGAG GAACTGGTAAACCAGGCC-3') from a clinical isolate NVT-1002. The amplified fragments were digested with restriction enzymes and cloned into the expression region of plasmid pET30a (Novagen). The pET30a-OmpK36 was used to express a 6His-OmpK36 protein as described below.

Recombinant OmpK36 protein expression, purification and detoxification

The pET30a-OmpK36 was transformed into BL-21 (DE3) competent cells. An overnight cell culture (3 mL) was grown at 37°C in the presence of 50 mg/L kanamycin. After transfer of the cell culture to 300 ml of Luria-Bertani medium, the cell suspension was allowed to reach an OD₆₀₀ of 0.7–0.9 before addition of IPTG (1 mM). Cells were grown for 4 h at 37°C and then centrifuged at 9000g for 30 min. Whole-cell lysates were prepared by sonication (Sonics Vibracell sonicator, 25% amplitude, pulsed 1 sec "on" and 2 sec "off" for a total of 5 min of "on" time, at 4 sec) in phosphate buffered saline with 6 M urea. The soluble protein fraction was then mixed with 8 mL of Ni²⁺ resins (Amersham) to capture the His-OmpK36 recombinant protein. The Ni²⁺ resins was then washed with 40 mL of wash buffer (10 mM imidazole, 6 M urea, 10% glycerol in phosphate buffered saline (PBS)) and eluted by wash buffer with 40 mM imidazole. The flowthrough was collected and dialyzed by PBS with 3M urea and 10% glycerol for 4 hr and then by PBS with 10% glycerol overnight. The endotoxin was removed by Pierce Detoxi-Gel endotoxin removal gel (Thermo Scientific, Rockford, IL) as described in user's manual. Expression of the His-OmpK36 protein was determined Western blot and the final protein concentration was measured by Bradford assay (Bio-Rad).

Culture of hepatoma HepG2 cells

HepG2 cells was cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS), incubated at 37°C , 5% CO₂ in a humidified incubator. The culture medium will be changed every day. Cells were grown to confluence in 25 cm² tissue flasks. Subsequently, the cells was trypsinized with trypsin/EDTA solution in phosphate-buffered saline (PBS), centrifuged and resuspended in fresh media. Before the experiment, cells were seeded onto the variant tissue culture dishes overnight.

Cell viability assay

Cell viability was detected by MTS assay, Cell titer 96[®] AQueous (Promega). In brief, 2×10^4 cells were seeded on to 48 well plates in 200 μl serum free media per well and incubated for 24 hrs. After variant treatments included of a multiplicity of infection (MOI) of 60

whole bacterial infection or OmpK36 recombinant protein, cells were washed with PBS 3 times, 20 μl MTS reagents were added to each well in the final volume of 100 μl and placed in incubator for 2 hours. The optical density was measured at 490 nm using a plate reader.

Statistical analysis

Student's t test was used for statistical analysis. Data represented as means \pm standard deviations. P values of less than 0.05 was considered statistically significant.

Results

Contribution of OmpK36 the *K. pneumoniae* induced liver abscess

Histological sections showed that remarkable injuries in liver were seen after 24 hours of injection with wild type *K. pneumoniae* (NVT-1002). More severe injuries were found at day 2 and day 3 (Figure 1). In the meanwhile, the injuries were almost abolished in mice injected with OmpK36-KO. This is suggested that OmpK36 plays an important role in *K. pneumoniae* induced liver abscess (Figure 1).

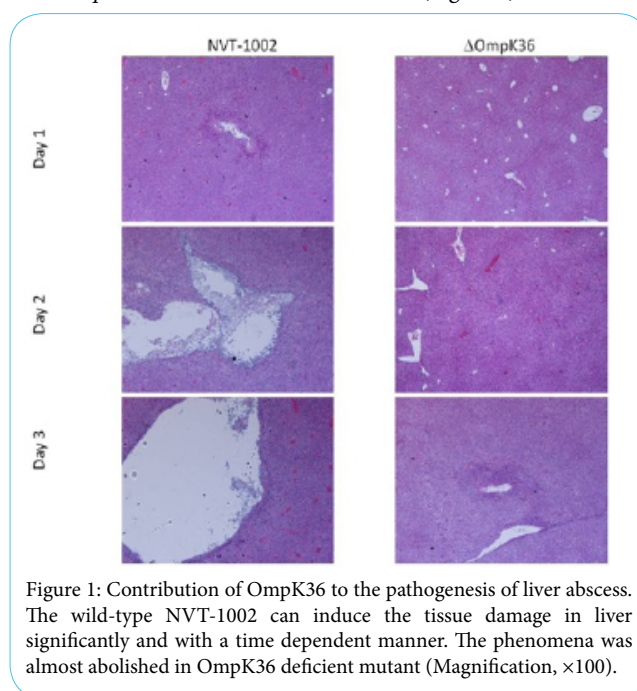


Figure 1: Contribution of OmpK36 to the pathogenesis of liver abscess. The wild-type NVT-1002 can induce the tissue damage in liver significantly and with a time dependent manner. The phenomena was almost abolished in OmpK36 deficient mutant (Magnification, $\times 100$).

Roles of OmpK36 in *Klebsiella pneumoniae* induced cytokines expression, included of TNF- α , IL- 1β , IL-6 and IL-10

Pro- and anti-inflammatory cytokines secretion in response to bacterial infection included IL- 1β , IL-6, IL-10 and TNF- α were detected by ELISA. The wild-type *K. pneumoniae* NVT-1002 elicited the secretions of all the cytokines both in plasma (Figure 2) and liver (Figure 3). It is notable that most of the elevated cytokines have the peaks at day 2. In contrast, the OmpK36 KO mutant only transiently enhanced IL- 1β expressions and the other cytokines were almost undetectable both in liver and serum.

Direct cytotoxic effects of OmpK36 recombinant protein and whole bacterial infection to HepG2 hepatoma cells

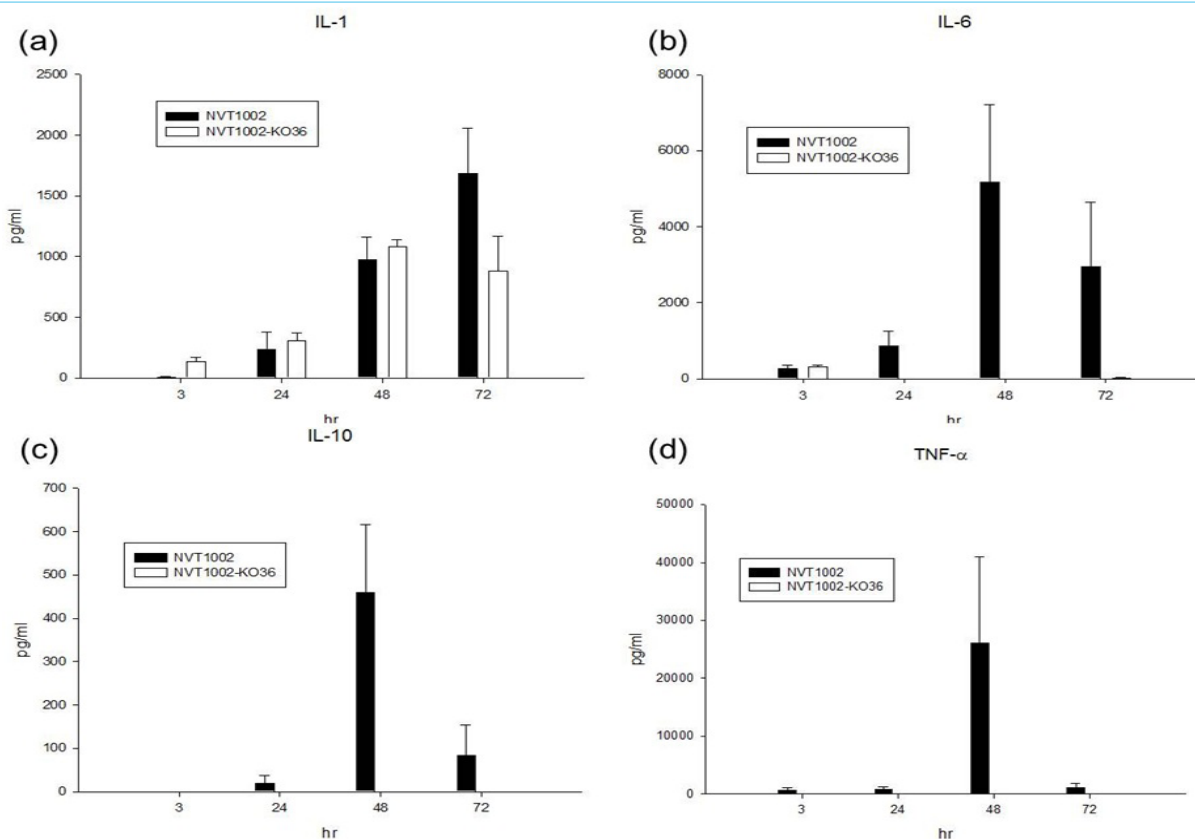


Figure 2: Role of OmpK36 in serum cytokines TNF- α , IL-1 β , IL-6 and IL-10 secretion. The wild-type NVT-1002 can elevate the serum cytokines significantly including TNF- α , IL-1 β , IL-6 and IL-10, while the Δ OmpK36 can only enhance IL-1 β expression. The value was mean \pm standard error of mean (SEM) at 3, 24, 48 and 72 hours of reaction, n=5.

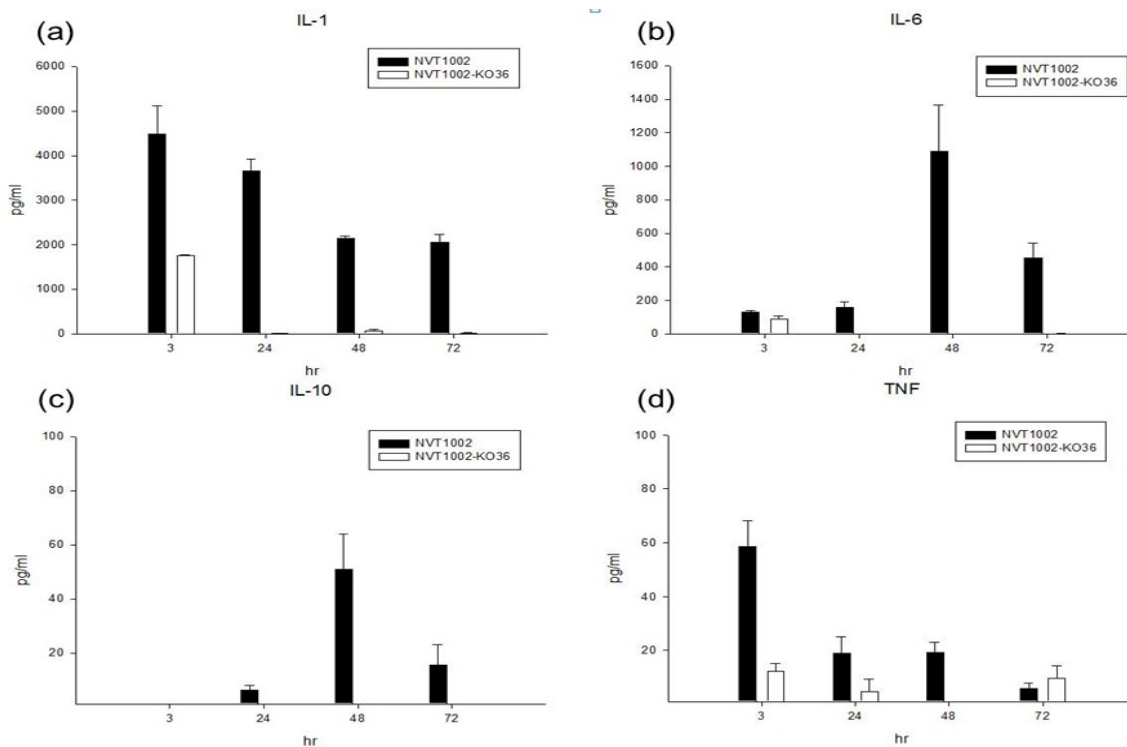


Figure 3: Role of OmpK36 in hepatic cytokines TNF- α , IL-1 β , IL-6 and IL-10 secretion. The wild-type NVT-1002 can elevate the hepatic cytokines significantly including TNF- α , IL-1 β , IL-6 and IL-10, while the Δ OmpK36 can only enhance IL-1 β expression. The value was mean \pm standard error of mean (SEM) at 3, 24, 48 and 72 hours of reaction, n=5.

HepG2 hepatoma cells were incubated with various concentrations of OmpK36 recombinant protein for 24 hours or infected with whole bacteria with a MOI of 60 for 2-6 hours. The viability of HepG2 cells were then detected by MTS. Results showed that no significant difference was detected in the toxicity to HepG2 cells (Figure 4a). Similar results were found in the toxicity of whole bacterial infection, the cell viability was not altered in the Δ OmpK36 group compared to the NVT-1002 group (Figure 4b).

demonstrated that high IL-10 / TNF- α ratio is associated with the mortality [30]. In this study, the serum TNF- α , IL-6 and IL-10 were elevated and have the peaks at day 2 after injection of wild type NVT-1002 (Figure 2). Similar results were found in hepatic IL-6 and IL-10 (Fig. 3). At the time point, severe injuries were observed in tissue sections (Figure 1). It is possible that the expressions of cytokines are associated with the pathogenesis of liver abscess. In the OmpK36 deficient group, both serum and hepatic IL-1 β were elevated, the other

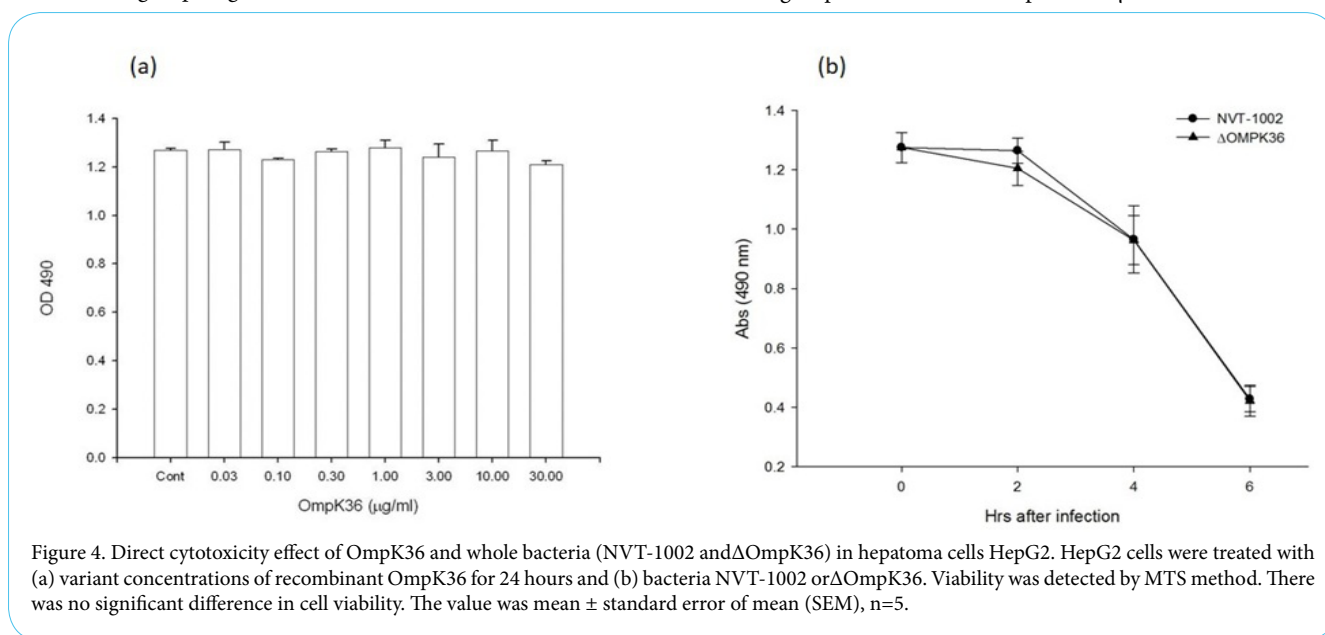


Figure 4. Direct cytotoxicity effect of OmpK36 and whole bacteria (NVT-1002 and Δ OmpK36) in hepatoma cells HepG2. HepG2 cells were treated with (a) variant concentrations of recombinant OmpK36 for 24 hours and (b) bacteria NVT-1002 or Δ OmpK36. Viability was detected by MTS method. There was no significant difference in cell viability. The value was mean \pm standard error of mean (SEM), n=5.

Discussion

Liver abscesses caused by *K. pneumoniae* were first reported in Taiwan [1,16]. The extra-hepatic complications spread by blood stream such as endophthalmitis, fasciitis and meningitis have been reported [1,17-19]. The syndrome was subsequently reported in Asia and America [20, 21]. To date, the syndrome is emerging world widely [22]. Several virulent factors of *K. pneumoniae* were described, including lipopolysaccharide, capsular polysaccharide, siderophore, resistance to phagocytosis and serum killing [23]. *K. pneumoniae* strains expressing capsular type K1 or K2 antigen are especially virulent. [7,24]. Previously, we first demonstrated that OmpK36 is a virulent factor of *K. pneumoniae*. However, the involvement of this porin in *K. pneumoniae* induced liver abscess progression is still unclear. The wild-type NVT-1002 used in this study is a serotype K1 clinical isolate which was isolated from patient with liver abscess. Injection with NVT-1002 intraperitoneally can induce severe hepatic injuries in experimental mice (Figure 1). On the contrary, the injuries were almost abolished in OmpK36 KO mutant group (Figure 1). Although this model cannot simulate the disease completely, it is still suggested that OmpK36 plays an important role in the pathogenesis of *K. pneumoniae* induced liver abscess.

Endogenous TNF- α s associated with severity of hemorrhagic and thrombotic lesions in organs, especially in the liver and kidney [25, 26]. IL-6 and IL-10 have the potential to inhibit TNF- α and IL- β expression from macrophage and peripheral monocytes [27, 28]. IL-6 also has the anti-inflammatory potential by activation of transcription-3 protein in hepatocytes [27]. The balance of the inflammatory and anti-inflammatory cytokines may determine the tissue damage and mortality [29]. A previous report has been

cytokines were almost undetectable (Figure 2 and Figure 3). This is evident that OmpK36 participate in the *K. pneumoniae* induced immune responses. However, the detail cellular and molecular mechanisms need further studies.

To identify the toxic effect of OmpK36, the *in vitro* cell model was used in this study. The HepG2 cells were treated with various concentrations of OmpK36 and the cell viability was detected by MTS method. Unexpectedly, the viability of HepG2 cells was not affected by recombinant OmpK36 protein (Figure 4a). Results of whole bacterial toxicity to HepG2 also showed that there is no significant difference was observed (Figure 4b). This is suggested that OmpK36 has no direct toxic effect to HepG2 cells. Previous study has been demonstrated that OmpK36 contributes to the resistance to phagocytosis, serum killing and the bacterial clearance in hepatocytes [15]. OmpK36 might prolong the duration of bacterial burden subsequently enlarge the injuries in organs such as liver and kidney.

The OmpK36 KO mutant used in this study should be with intact lipopolysaccharide and capsule. At least, the process of knock out is not affecting any target genes about LPS or capsule. Since the toxic effect is not from OmpK36 itself, another putative mechanism is that the expression of OmpK36 may influence the structure of other virulent factors such as LPS and capsule [31-33], the surface inflammatory inducer(s). However, the detail molecular mechanisms need more evidences.

In brief, OmpK36 contributes to the virulence of *K. pneumoniae*. The protein participates in the pathogenesis of *K. pneumoniae* induced liver abscess. The toxic effect of OmpK36 is mediated by enhancing other virulent factors possibly. The molecular mechanism(s) need further studies.

Competing Interests

The authors declare that they have no competing interests.

Funding

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References

- Liu YC, Cheng DL, Lin CL (1986) *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch Intern Med 146: 1913-1916.
- Yeh KM, Kurup A, Siu LK, Koh YL, Fung CP, et al. (2007) Capsular serotype K1 or K2, rather than magA and rmpA, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. J Clin Microbiol 45: 466-471.
- Fang FC, Sandler N, Libby SJ (2005) Liver abscess caused by magA+ *Klebsiella pneumoniae* in North America. J Clin Microbiol 43: 991-992.
- Karama EM, Willermain F, Janssens X, Claus M, Van den Wijngaert S, et al. (2008) Endogenous endophthalmitis complicating *Klebsiella pneumoniae* liver abscess in Europe: case report. Int Ophthalmol 28: 111-113.
- Keynan Y, Karlowsky JA, Walus T, Rubinstein E (2007) Pyogenic liver abscess caused by hypermucoviscous *Klebsiella pneumoniae*. Scand J Infect Dis 39: 828-830.
- Cheng KS, Tang HL, Hsu CH, Lai HC, Yu CJ, et al. (2007) A clinical survey of *Klebsiella pneumoniae* virulence and genotype in pyogenic liver abscess. Adv Ther 24: 589-593.
- Lin JC, Chang FY, Fung CP, Xu JZ, Cheng HP, et al. High prevalence of phagocytic-resistant capsular serotypes of *Klebsiella pneumoniae* in liver abscess. Microbes Infect 6: 1191-1198.
- Yeh KM, Chang FY, Fung CP, Lin JC, Siu LK, et al. (2006) magA is not a specific virulence gene for *Klebsiella pneumoniae* strains causing liver abscess but is part of the capsular polysaccharide gene cluster of *K. pneumoniae* serotype K1. J Med Microbiol 55: 803-804.
- S J Cryz Jr, Fürer F, Germanier R (1984) Experimental *Klebsiella pneumoniae* burn wound sepsis: role of capsular polysaccharide. Infect Immun 43: 440-441.
- Domenico P, Johanson WG Jr, Straus DC (1982) Lobar pneumonia in rats produced by clinical isolates of *Klebsiella pneumoniae*. Infect Immun 37: 327-335.
- Highsmith AK, Jarvis WR (1985) *Klebsiella pneumoniae*: selected virulence factors that contribute to pathogenicity. Infect Control 6: 75-77.
- Mizuta K, Ohta M, Mori M, Hasegawa T, Nakashima I, et al. (1983) Virulence for mice of *Klebsiella* strains belonging to the O1 group: relationship to their capsular (K) types. Infect Immun 40: 56-61.
- Hernández-Allés S1, Conejo Md, Pascual A, Tomás JM, Benedí VJ, et al. (2000) Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. J Antimicrob Chemother 46: 273-277.
- Martínez-Martínez L, Pascual A, Hernández-Allés S, Alvarez-Díaz D, Suárez AI, et al. (1994) Roles of beta-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. Antimicrob Agents Chemother 43: 1669-1673.
- Chen JH, Siu LK, Fung CP, Lin JC, Yeh KM, et al. (2010) Contribution of outer membrane protein K36 to antimicrobial resistance and virulence in *Klebsiella pneumoniae*. J Antimicrob Chemother 65: 986-990.
- Yang CC, Yen CH, Ho MW, Wang JH (1995) Comparison of pyogenic liver abscesses caused by *Klebsiella pneumoniae* and non-*K. pneumoniae* pathogens. J Formos Med Assoc 94: 232-237.
- Saccante, M (2006) *Klebsiella pneumoniae* liver abscess, endophthalmitis, and meningitis in a man with newly recognized diabetes mellitus. Clin Infect Dis 29: 1570-1571.
- Hu BS, Lau YJ, Shi ZY, Lin YH (1999) Necrotizing fasciitis associated with *Klebsiella pneumoniae* liver abscess. Clin Infect Dis 29: 1360-1361.
- Cheng HP, Siu LK, Chang FY (2003) Extended-spectrum cephalosporin compared to cefazolin for treatment of *Klebsiella pneumoniae*-caused liver abscess. Antimicrob Agents Chemother 47: 2088-2092.
- Rahimian J, Wilson T, Oram V, Holzman RS (2004) Pyogenic liver abscess: recent trends in etiology and mortality. Clin Infect Dis 39: 1654-1659.
- Pastagia M, Arumugam V (2008) *Klebsiella pneumoniae* liver abscesses in a public hospital in Queens, New York. Travel Med Infect Dis 6: 228-233.
- Struve C, Bojer M, Nielsen EM, Hansen DS, Krogfelt KA, et al. (2005) Investigation of the putative virulence gene magA in a worldwide collection of 495 *Klebsiella* isolates: magA is restricted to the gene cluster of *Klebsiella pneumoniae* capsule serotype K1. J Med Microbiol 54: 1111-1113.
- Podschun R, Ullmann U (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11: 589-603.
- Yeh KM, Chiu SK, Lin CL, Huang LY, Tsai YK, et al. (2016) Surface antigens contribute differently to the pathophysiological features in serotype K1 and K2 *Klebsiella pneumoniae* strains isolated from liver abscesses. Gut Pathog 8: 4.
- Nakajima Y, Momotani E, Takahashi H, Ishikawa Y, Ito T, et al. Endogenous tumor necrosis factor (TNF) production and modification of pathological lesions in experimental *Escherichia coli* endotoxemia of piglets. Vet Immunol Immunopathol, 45: 45-54.
- Nakajima Y, Mikami O, Yoshioka M, Arai S, Miura K, et al. (2000) Involvement of apoptosis in the endotoxemic lesions of the liver and kidneys of piglets. J Vet Med Sci 62: 621-626.
- Teoh N, Field J, Farrell G (2006) Interleukin-6 is a key mediator of the hepatoprotective and pro-proliferative effects of ischaemic preconditioning in mice. J Hepatol 45: 20-27.
- Armstrong L, Millar AB (1997) Relative production of tumour necrosis factor alpha and interleukin 10 in adult respiratory distress syndrome. Thorax 52: 442-446.
- Charavaryamath C, Janardhan KS, Caldwell S, Singh B (2006) Pulmonary intravascular monocytes/macrophages in a rat model of sepsis. Anat Rec A Discov Mol Cell Evol Biol 288: 1259-1271.
- Gogos CA, Drosou E, Bassaris HP, Skoutelis A (2000) Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. J Infect Dis 181: 176-180.
- Soltes GR, Schwalm J, Ricci DP1, Silhavy TJ (2016) The Activity of *Escherichia coli* Chaperone SurA Is Regulated by Conformational Changes Involving a Parvulin Domain. J Bacteriol 198: 921-929.
- Skorek K, Raczowska A, Dudek B, Miętka K, Guz-Regner K, et al. (2013) Regulatory protein OmpR influences the serum resistance of *Yersinia enterocolitica* O:9 by modifying the structure of the outer membrane. PLoS One 8: e79525.
- Davies RL, MacCorquodale R, Caffrey B (2003) Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. Vet Microbiol 91: 169-182.