Inactivation of Dipeptidyl Peptidase IV Attenuates the Virulence of *Streptococcus suis* Serotype 2 that Causes Streptococcal Toxic Shock Syndrome

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Abstract Di-peptidyl peptidase IV (DPP IV), originally recognized as CD26 in eukaryotic cells, is distributed widely in microbial pathogens, including *Streptococcus suis (S. suis)*, an emerging zoonotic agent. However, the role of DPP IV in *S. suis* virulence remains unclear. Here, we identified a *dpp IV* homologue from highly invasive isolate of *S. suis* 2 (SS2) causing streptococcal toxic shock syndrome (STSS). Enzymatic assays reproduced its enzymatic activity of *dpp IV* protein product as a functional DPP IV, and ELISA analysis demonstrated that SS2 DPP IV can interact with human fibronectin. An isogenic SS2 mutant of *dpp IV*, Δ*dpp IV*, was obtained by homologous recombination. Experimental animal infection suggested that an inactivation of *dpp IV* attenuates greatly its high virulence of Chinese virulent strains of SS2. Functional complementation can restore this defect in SS2 pathogenicity. To our knowledge, it may confirm, for the first time, that DPP IV contributes to SS2 virulence.

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Introduction

Streptococcus suis (S. suis) can be divided into 35 serotypes (1–34 & 1/2), on the basis of difference of capsule antigens [25]. Of particular note, *S. suis* serotype 2 (*S. suis* 2, SS2) has emerged into a highly infectious entity that are responsible for a collection of sporadic cases [6, 19, 22] and even outbreaks [27, 29, 31] of human SS2 infections. A new severe disease-form, streptococcal toxic shock syndrome (STSS), which is previously found in patients due to *Streptococcus pyogenes* (GAS) infections, was observed in two large-scale outbreaks of human SS2 epidemics in China [24, 27]. Experimental infections of piglets showed these isolated SS2 strains are highly pathogenic, implying that they have evolved some mechanisms to become a super-bug and then challenge public health [27]. Comparative genomics of these deadly strains of Chinese SS2 revealed a potential pathogenic island (PAI), named as 89K, which is specific to Chinese virulent strain [5]. From this unique region, 89K, a two component signal transduction system (TCSTS), *SalK/SalR* recently was identified to be essential for full virulence of STSS-causing Chinese SS2 strains [17]. Additionally, a trans-peptidase, called Sortase A [28], was demonstrated to be associated with high pathogenicity of Chinese SS2 strains. More intriguingly, an orphan regulator CovR [20] was suggested to down regulate virulence of these STSS-causing pathogens.

Towards better understanding of SS2 pathogenesis, our laboratory has initiated relevant genetic/immunological

studies and gained a couple of insights into SS2 virulence [5, 7, 8, 17, 20, 28]. Based on the decoded SS2 genome sequences, a collection of protective antigens were identified [18]. In the complex interplay between microbial pathogens and its host, bacterial proteases are recognized as potential virulence-associate factors with contribution to tissue degradation and impairment of the host immune system [12, 13]. Di-peptidyl peptidase IV (DPP IV) is a serine protease that cleaves X-Pro/Ala di-peptide from the N-terminus of proteins. This protease has been suggested to be widely distributed in eukaryotes and bacteria. Previous studies showed that eukaryotic DPP IV is involved in disease progression, such as diabetes [10, 11]. Genetic data from *Porphyromonas gingivalis* also demonstrated that DPP IV contributes to its pathogenesis [15, 16]. Retrospectively, four major proteases including DPP IV were ever found in the enzoonotic agent, *S. suis*. The DPP IV protein produced by *S. suis* can be divided into two forms: one is cell bound, the other is extracellular [12]. Although the encoding gene of DPP IV and its relevant enzymatic characteristics has been known for several years, its role in SS2 pathogenesis is still unclear [13].

In this study, we cloned *dpp IV* gene from Chinese strains of SS2 that causes STSS, and unexpectedly found that the protein product can bind to human fibronectin. More interestingly, we observed that inactivation of *dpp IV* gene impairs its virulence in STSS-causing SS2. These findings may extend our understanding towards molecular pathogenesis of the fatal Chinese SS2 strains, and provide basis for vaccine development targeting an interaction between SS2 and its host.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids were listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI) medium or plated on THB agar with 5% (vol./vol.) sheep blood. *Escherichia coli* strains were maintained using Luria Broth (LB) liquid medium/LB agar. Antibiotics were used as follows: spectinomycin (Spc), 100 μg/ml; ampicillin (Amp), 100 μg/ml.

Preparation and Evaluation of SS2 DPP IV

DPP IV encoding region was amplified from strain 05ZYH33 using the primers of *dpp IV*-1 & *dpp IV*-2 (Table 2), and then cloned into pET32a via *Bam*HI and *Xho*I restriction sites, resulting in the recombinant plasmid pET32a-*dpp IV*. After verification by direct DNA sequencing, it was transformed into *E. coli* BL21 for over

expression of DPP IV protein. The production and purification was performed as Feng et al. [7, 8] previously described. The enzymatic activity of SS2 DDP IV protein was assayed as Jobin et al. [13] suggested previously, and its immunogenicity/antigenicity was evaluated using western blot with convalescent-phase serum from piglets infected with SS2 [7].

Dose-Dependent Inhibition of SS2 Adherence to Hep-2 Cells

SS2 adherence to pharyngeal cells was determined as described by Charland et al. [4]. The human laryngeal epithelial cell line Hep-2 were cultured at 37°C and 5% CO_2 in RPMI 1640 medium with 10% heat-inactivated foetal bovine (Gibco-BRL) without antibiotics overnight, when grown to confluence in 24-well tissue culture plates (0.5×10^6–1.0×10^6 cells/well), infected with bacteria at a MOI (multiplicity of infection) of 1:50 in RPMI 1640 in the presence of increasing amounts of DPP IV (5,10 and 15 μg). After 3 h incubation at 37°C in 5% CO_2, unbound bacteria were removed by washing wells with PBS and treated with lysis buffer (RPMI 1640 containing 0.1% trypsin, 1.0% Triton X-100), then the resulting lysates were appropriately diluted in sterile PBS. The actual numbers of Hep-2 cells associated SS2 were determined by plating serial dilutions of lysates in triplicates on blood agar plates and incubated at 37°C for 24 h counting cfus. All experiments were performed in duplicate wells and repeated at least thrice. In each experiment, wells containing only cells and only bacteria were used as controls.

ELISA

To probe possible interaction between DPP IV and human fibronectin, enzyme-linked immuno-sorbent assay (ELISA) was employed [7]. In brief, micro-titer plates (96 wells, flat bottom, polystyrene) (Corning Costar, Cambridge, Mass) were coated with 1 μg of ECM protein (human plasma fibronectin catalogue no. F-0895; Sigma-Aldrich) diluted with phosphate-buffered saline (PBS) and incubated for 12 h at 4°C. The wells coated with 1 μg of casein (Wako) functioned as the negative control. Removed the supernatant, wells were washed thrice with PBS, and then blocked with 10 μg heat-denatured casein for 2 h at room temperature. After washing thrice with PBST (PBS with 0.1% Tween 20), 100 μl of purified DPP IV protein (diluted in PBS) was added to each well and incubated for 2 h at 37°C. Following three times of washing with PBST, wells were incubated with 100 μl of rat anti-DPP IV serum for 2 h at room temperature. Similarly, wells were incubated with 100 μl of alkaline phosphatase-conjugated goat anti-rat immunoglobulin G (IgG) (Zymed, South San Francisco,

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source/reference
Strains		
S. suis serotype 2		
05ZYH33	Virulent strain isolated from a dead patient with STSS	Lab collection
Δddp IV	Isogenic ddp IV mutant of strain 05ZYH33, Spc^R	This study
05HAS68	Avirulent strain isolated from a healthy pig	Lab collection
CΔddp IV	Complemented strain of dpp IV, Spc^R, Cm^R	This study
E. coli		
DH5α	Host for cloning vector	Promega
BL21	Host for protein expression	Lab collection
Plasmids		
pMD18-T	TA cloning vector, Amp^R	TaKaRa
pMD-dpp IV-spc	Recombinant plasmid designed for knockout of dpp IV; Amp^R; Spc^R	This study
pET32a-dpp IV	Expression vectors; Amp^R	This study
pSET1	E. coli–S. suis shuttle vector, Cm^R	[26]
pSET1-dpp IV	pSET1 containing an intact dpp IV gene and its upstream promoter, Cm^R	This study

*Amp^R, ampicillin resistant; Spc^R, spectinomycin resistant; Cm^R, chloromycetin resistant

Table 2 Primers used in this work

Primers	Sequence (5′–3′)	Target gene or location
dpp IV-1	5′-GCGGATCCATGCGCTTTAATCAATTTTCTTCATAAAAAAAGAGAC-3′	To amplify the ddp IV gene
dpp IV-2	5′-GCCTCGAGTTAGTGCGGTAAAATTAACTGTG-3′	
LA	5′-AAGTGCCATTTCCTGACAGC-3′	A fragment with ddp IV
RA	5′-GTTACAGGCAGTTCAATGCT-3′	
LU	5′-GAAACCTTTGTCAGAAAG-3′	Outside fragment of ddp IV
RD	5′-AGCAAGAGGCCCTCCGTT-3′	
Spc-F	5′-AGGCCTGTTCGTGAATACATGTTATA-3′	Spc^R gene cassette
Spc-R	5′-AGGCCTGTTTTCTAAAATCTGAT-3′	
CΔdpp IV -F	5′-GCGGATCCCGTAAAATAGGGACTATAGCTTC-3′	The dpp IV gene and its upstream promoter
CΔdpp IV -R	5′-GCGAATTCTATTAGTGCGGTAAAATTAACTGTGA-3′	

Calif.) for 2 h at room temperature. Finally, the samples were developed with O-phenylenediamine as a substrate (Amresco) and H_2O_2 (Sigma) as the oxidation agent, and the absorbance scores were measured at 490 nm in a micro-plate reader (Model 500; Bio-rad).

Cell Adherence Assays

Human laryngeal epithelial cell line, Hep-2 was used here. Bacteria were grown to mid-log phase in THB ($OD_{600} = 0.6$), washed twice with PBS, and then re-suspended in Eagle's medium. Cells were infected at a multiplicity of infection of 10 (bacteria per cell) for 2 h at 37°C in 5% CO_2. The monolayer of cells was then washed thrice with PBS, and disrupted by the addition of 0.3 ml of sterile deionized ice-cold water and repeated pipetting. The 100 μl-aliquots diluted 1:10 or 1:100 in PBS were used for quantitative plating. The percent adherence was calculated as the formula below, (cfu on plate/cfu in original inoculum) × 100.

Construction of dpp IV Inactivation Mutant, Δdpp IV

The DNA fragment covering partial sequence of dpp gene was amplified from SS2 05ZYH33 with primers LA & RA (Table 2), and cloned into pMD18-T vector (Takara), generating the recombinant plasmid pMD-dpp IV. Then Spc^R gene cassette was designed to insert into the coding region of dpp IV gene carried in the pMD-dpp IV via a unique restriction site, Stu I, generating the dpp IV knock-out

plasmid, pMD-*dpp IV-spc*. Subsequently, the acquired knock-out plasmid was electroporated into SS2 competent cells [28], and the transformants were screened on THB plates complemented with 100 µg/ml of spectinomycin. To confirm the *dpp IV* knock-out mutant, multiple lines of approaches were utilized, such as PCR, RT-PCR and Southern blot.

Complementation of the *dpp IV* Inactivation

For complementation assay, the entire *dpp IV* gene and its upstream promoter was amplified using primers C∆*dpp IV*-F and C∆*dpp IV*-R, which contained *Bam*HI and *Eco*RI sites, respectively. After double digestion, it was cloned into the shuttle vector, pSET1 [26] via *Bam*HI/*Eco*RI sites, generating the recombinant plasmid, pSET1-*dpp IV*. Then, this plasmid was electroporated into the ∆*dpp IV* mutant to obtain the complemented strain, C∆*dpp IV*. Further verification was performed using PCR and RT-PCR.

Experimental Animal Infections

To evaluate the virulence of the ∆*dpp IV* and C∆*dpp IV*, the mice model was adopted. The bacterial growth phase was judged by measuring the optical densities, and adjusted to the desired concentration in PBS. All mice were challenged with subcutaneous injections of 1 ml of bacterial suspension. Mice were examined daily to assess their general health status, as well as the presence and location of lesions. The wild-type strain 05ZYH33 [17, 28] and an avirulent strain 05HAS68 [28] served as positive control and negative control, respectively.

Protection Assay

Purified DPP IV proteins were used to immunize mice. A total of 30 mice were divided into 3 groups (10 mice/group). Group 1 was not immunized, group 2 was for immunization with DPP IV, and group 3 is immunized with PBS. Female 4-week-old BALB/c mice were primed by a subcutaneously injection of 25 µg DPP IV proteins mixed with complete Freund's adjuvant, and two boosted every 2 weeks with a subcutaneously injection with 25 µg DPP IV proteins and incomplete Freund's adjuvant, respectively. Three days after the last booster, the titre of antibody against DPP IV was in high level. Then, the protective assays were tested. 05ZYH33 was grown for 6 h in THS and adjusted to the density of 10^8 cfu/ml in THS media (THB containing 10% foetal bovine serum, 10^8 cfu represents the minimal lethal dose challenge infection) [2]. Each mouse was injected with 10^8 cfu of *S. suis* 05ZYH33. Mice were recorded for mortality during a period of 7 days.

Results

Enzymatic Properties of SS2 DPP IV Protein

The coding region of SS2 *dpp* IV gene is 2,265 nucleotides long, encoding DPP IV protein of 755 amino acids with an estimated molecular mass of ~ 87 kDa. To test if the over-expressed DPP IV protein is functional in vitro, a series of enzymatic assays were performed. Using Gly-Pro-pNA (Sigma) as substrate, the K_m and V_{max} values of the recombinant protein were estimated to be 0.3 mM and 40 µmol mg^{-1} min^{-1}, respectively (Fig. S1A, B). The range of optimum pH of the enzyme was 6.5–8.5, with the highest activity at pH 8.0 (Fig. S1C). Optimal temperature of reaction is about 37°C (Fig. S1D), and the result is similar to that previously described by Jobin et al. [12, 13]. Moreover, 20% of the enzyme activity remained, after incubation at 50°C for 30 min.

SS2 DPP IV Binds to Human Fibronectin

The visualized size of over-expressed DPP IV protein fused with an N-terminal His-tag and Trx-tag in SDS-PAGE behaves in much agreement with the above prediction result, about 105 kDa (not shown). The result of western blot using convalescent-phase swine sera showed that this protein is of antigenicity/immunogenicity, implying its expression is associated with SS2 infections (Fig. 1A). We also examined the binding of DPP IV to human fibronectin. Unexpectedly, ELISA analyses suggested that DPP IV can bind to immobilized human fibronectin, but not for casein, a negative control. In particular, the dose-dependent inhibition of SS2 adherence to Hep 2 cells was determined in the presence of increasing amounts of purified DPP IV, implying this interaction is dependent on the amount of DPP IV protein used (Fig. 1B, C). This finding may show some clues to SS2 pathogen adherence to host cell and even its invasion.

Construction of ∆*dpp IV* Strain and Functional Complementation

To further investigate its physiological roles of SS2 DPP IV in vivo, we developed a *dpp IV*-deficient mutant of SS2 05ZYH33, refers as ∆*dpp IV*, using the strategy of homologous recombination (Fig. S2A). After several rounds of screening, we found a suspected mutant from more than 100 transformants with resistance to spectinomycin. The results of PCR-based detection showed that the *dpp IV* gene has been disrupted by the introduced spectinomycin-resistant cassette successfully in this mutant candidate (Fig. S2B, C). Furthermore, the results of Southern blot suggested that (1) the interested band in

Fig. 1 Immunological characteristics of SS2 DPP IV protein. **A** Western blot analysis of DPP IV protein using convalescent-phase swine sera and SPF swine sera. +: convalescent-phase swine sera; –: SPF (specific-pathogen-free) swine sera. **B** Role of DPP IV in *S. suis* 2 adherence to Hep 2 cells. The pharyngeal cell-associated *S. suis* 2 was counted as colony-forming units (cfu). Dose-dependent inhibition of *S. suis* 2 adherence to Hep 2 cells was determined in the presence of increasing amounts of purified DPP IV protein. * $P < 0.05$. **C** Dose-dependent binding of DPP IV protein to human fibronectin
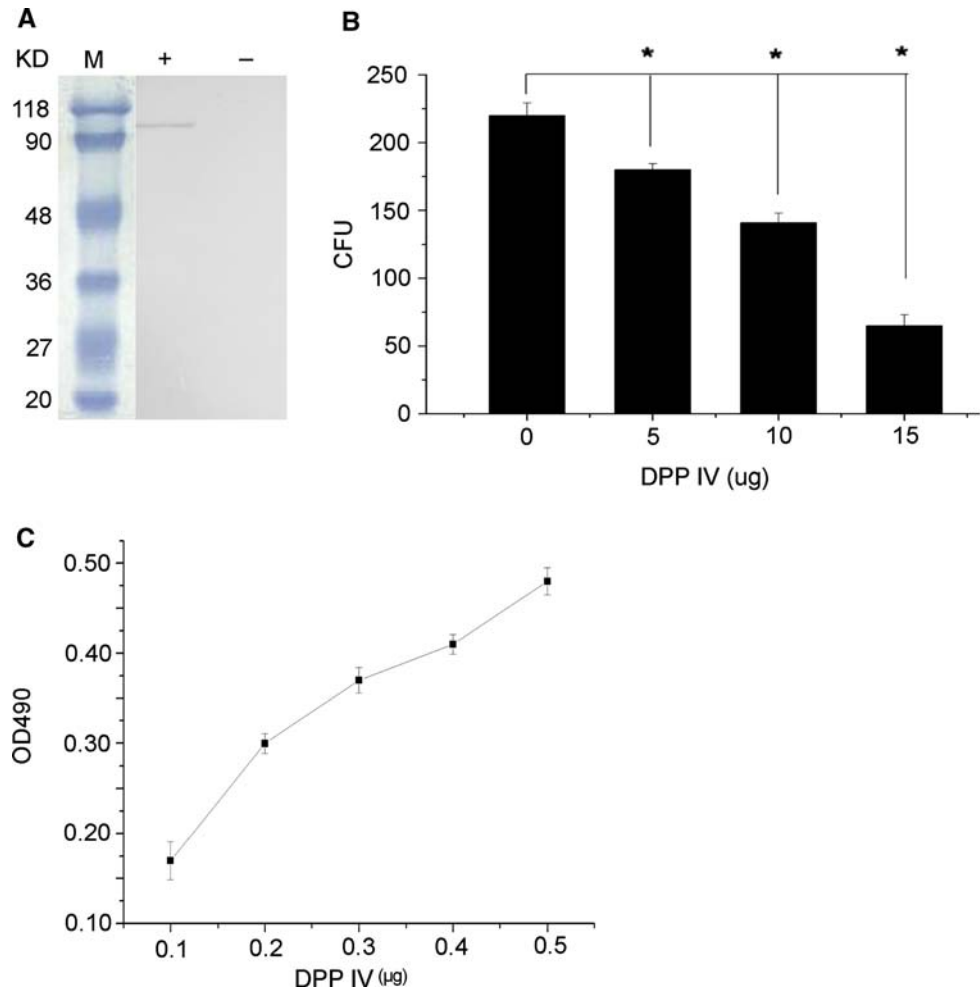
mutant is much bigger than that of parental strain, using the probe of *dpp IV*; (2) when spectinomycin-resistant cassette serves as probe, the band of expected size was present in the mutant strain, while none is in the parental strain (Fig. S2C). RT-PCR demonstrated that the expression of *dpp IV* gene can be detected in the parental strain and the complemented strain, but not in the mutant strain (Fig. S2D). Collectively, we can conclude that an isogenic mutant of *dpp IV* gene has been successfully constructed.

Defect of SS2 Adhesion Capability due to Disruption of *dpp IV* Gene

The disruption of *dpp IV* gene fails to show significant effect on the growth, indicating it is not essential for bacterial survival in rich media (not shown). Similarly, haemolytic activity of Δ*dpp IV* also behaves similarly to that of wild strain, 05ZYH33 (not shown). To test whether the *dpp IV* gene plays any role in bacterial adherence, Hep-2 cell line was utilized. Unexpectedly, the Δ*dpp IV* mutant showed much less adherence to Hep-2 cell line compared with the wild type strain (Fig. 2A). Considering that DPP

IV protein can interact with human fibronectin, it is reasonable that inactivation of *dpp IV* gene can alter significantly its cellular adhesion ability. Thus, *dpp IV* gene is proposed to play a role at the initial step in SS2 infections.

Role of *dpp IV* Gene in SS2 Pathogenicity

To further assess the contribution of *dpp IV* gene to SS2 virulence, mice infection models were used. Totally, 40 mice were divided into 4 groups (10 mice/group); group 1 for virulent strain, group 2 for the mutant Δ*dpp IV*, group 3 for the complemented strain, CΔ*dpp IV*, and group 4 for the avirulent strain, 05HAS68. The challenge dose is determined to be 1×10^8/mouse. In the first group, 3 of 10 mice inoculated with wild-type 05ZYH33 died in 8 h (all the other animals displayed the cachectic appearance), and the other three were dead by 16 h, all of the animals were not exempt by 20 h. Also, in third group, 2 of mice inoculated with complemented strain, CΔ*dpp IV* died in 8 h, and the other three were dead by 16 h, only one mouse was survived by 20 h. Thus, we concluded that the mortality between wild-type strain and complemented strain were
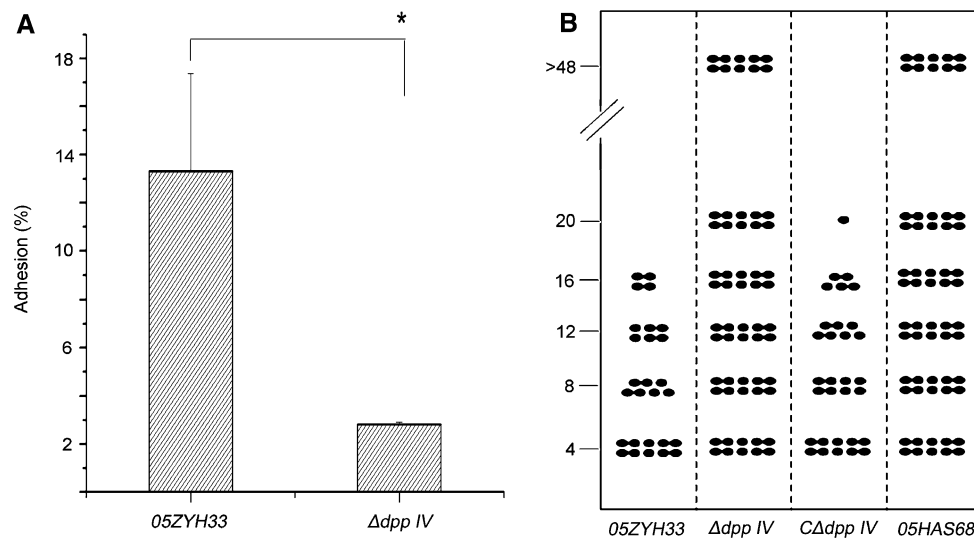
Fig. 2 Role of *dpp IV* in SS2 pathogenicity. **A** Adhesion capability is impaired by disruption of *dpp IV* in *Streptococcus suis* 2. Results were determined after a 1-h co-incubation of *S. suis* strains with human epithelial (Hep-2) cells at a MOI of 10:1, followed by an extensive washing of non-adherent bacteria, and cells lysis to retrieve 100 μl aliquots of total cell-associated bacteria for viable plate counts. Results shown are the mean ± SD of three independent experiments. * $P < 0.05$. **B** Mouse infection assays. Groups of 10 SPF-mice were challenged intravenously with approximately 10^8 cfu of the indicated strains. The survival time (hours) of each mouse is indicated. Each datum point represents one mouse

similar (Fig. 2B). In contrast, mice treated with the Δ*dpp IV* mutant behave normal and healthy. It seems to be completely similar to those inoculated with avirulent strain. All the mice survived post 48 h after the bacterial challenge (Fig. 2B). These results demonstrated that inactivation of *dpp IV* impairs completely virulence of SS2 suggesting that the di-peptidase activity is essential for pathogenesis of STSS-causing SS2.

The Capacity of the Protection for DPP IV

The capacity of the protection for DPP IV was evaluated with the protection rate. After injected with the highly pathogenic strain 05ZYH33, the mortality of the challenged mice was observed for a week. As shown in Table 1, we could see that 10 of 10 mice of both the group immunized with none and the group immunized with PBS (negative control) were died in 7 days. In contrast, none was died in the group of immunized with the purified protein DPP IV. Compared with the group immunized with PBS, the group immunized with DPP IV showed 100% survival proportion ($P < 0.001$) (Table 3).

Discussion

S. suis is an important worldwide swine pathogen [25] and an emerging human threat [22, 29]. Complete understanding of its pathogenesis is critical to develop effective approaches to combat against its severe infections. Chinese variants of SS2 seemed to be highly invasive and much more pathogenic [24, 27]. Recently, Dr. Xu's group proposed that 05' epidemic SS2 strains in China possess the ability to stimulate the production of massive amounts of pro-inflammatory cytokines, which may account for the manifestation of STSS in those patients infected with SS2 [30]. More interestingly, our collaborator showed evidence that heterogeneous SS2 populations are circulating in China now [6]. Eukaryotic dipeptidyl peptidase IV is known be implicated into a series of biological functions with T-cell activation included [21]. The homologues of *dpp IV* gene were also found to be in several bacteria, such as *Lactobacillus helveticus* [1], *Streptococcus gordonii* [9], *P. gingivalis* [15, 16], etc. However, only the *dpp IV* locus in *P. gingivalis* has been reported to be a virulence determinant thus far. Fibronectin-binding protein of *Staphylococcus aureus* is thought to act as an invasin through fibronectin-dependent interaction with α5,β1-integrin [23]. *P. gingivalis* invading the connective tissue, mainly via a paracellular pathway, attaches to the ECM through the interaction of fibronectin and DPPIV and establishes colonization [14]. In 1997, Beauvais et al. [3.] found the ability of the DPP IV to bind collagen and hydrolyze some biological peptides, suggesting a possible role during lung invasion by *Aspergillus fumigatus*.

Although the *dpp IV* gene was identified from *S. suis* for several years, its physiological/pathological roles remains unclear. In this study, we aimed to evaluate its possibility of being a virulence associated factor using the strategy of gene knock-out together with infections of experimental

Table 3 Protection assays in mice

Group	Dose of injection (cfu)	No. of mice killed/ No. of mice inoculated	Protection efficiency (%)
1	10^8	10/10	ND
2	10^8	0/10	100*
3	10^8	10/10	0

1, Group of without immunity (+). 2, Group of immunized with DPP IV; 3, Group of immunized with PBS (−)

* $P < 0.001$

ND, not determined

animals. Consequently, we observed the following two new findings: (1) the purified DPP IV exhibits capability of binding to human fibronectin, which can be implicated for the adherence of SS2 to host cell surface, and (2) the disruption of *dpp IV* gene impairs full virulence of *S. suis* 05ZYH33 strain in the mice model. In addition, the SS2 DPP IV shows robust antigenicity and has also been successfully developed a DPP IV-based ELISA method for monitoring the SS2 infections in swine populations and suspected-patients (not shown).

The search for prevention/therapeutics approaches of SS2-related disease is always of much interest worldwide. Therefore, the potential of SS2 DPP IV as subunit-vaccine was being addressed in our laboratory. Preliminary results suggested it hopefully serve as a protective antigen, in part similar to that observed recently in SS2 enolase (data submitted). These findings may enrich the knowledge of SS2 pathogenesis, especially Chinese virulent strains, and facilitate to develop new strategies against the challenge of deadly SS2 infections.

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