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Effect of VirD4 on gastric epithelial-1 cells and its mechanism

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Abstract: The gene of *Helicobacter pylori* can encode three to four type IV secretory systems, of which a new gene region has been found in the *H. pylori* plasticity region. The coding products of this region can form a new T4SS named tfs3, but its function is unclear. This study investigated the effect of VirD4 recombinant protein in the tfs3 secretory system of the *H. pylori* clinical strain SBK on GES-1 cells. We observed changes in cell morphology after VirD4 treatment. Further analysis indicated that VirD4 increased inflammation by increasing the activation of NF-κB. VirD4 can also inhibited proliferation, and induced migration of cells. Moreover, VirD4 caused apoptosis in GES-1 cells in caspase and ERK1/2/Ras dependent signaling events. Our study laid a foundation for further research on the biological function of VirD4 and the detection and treatment of *H. pylori*-related diseases.

Introduction

Helicobacter pylori is a common pathogenic bacterium in the human digestive system (Backert *et al.*, 2004; Chmiela and Gonciarz, 2017). *H. pylori* infection often causes chronic active gastritis, peptic ulcers, and can develop into malignant tumors of the digestive system (Sanders and Peura, 2002). The type IV secretory system (T4SS) is one of the secretory and transporting mechanisms of *H. pylori*'s pathogenic factors and causes the proliferation of host cells, promotes the secretion of cytokines, and facilitates bacterial colonization and persistent infection (Naumann *et al.*, 2017). In addition to the cag pathogenicity island and comB locus that encode the different T4SSs of *H. pylori*, the plasticized zones of the *H. pylori* genome, of which *tfs3* is one, can also encode T4SS (Hofreuter *et al.*, 1998; Kersulyte *et al.*, 2003; Tegtmeyer *et al.*, 2011).

The cluster size of the *tfs3* gene is 16.3 KB, and the four coding genes virB4, virB7, virB11, and VirD4 are homologous with the well-studied virB/D4 TFSS of the plant pathogen *Agrobacterium tumefaciens* (Alvi *et al.*, 2007). Furthermore, it became clear that tfs3 and tfs4 genes are not restricted to the original "plasticity zones". They are organized together with further genes as genome islands in many other genomic locations (Fischer *et al.*, 2020). The function of the T4SS encoded by *tfs3* remains unclear. A possible role of Tfs3 has been suggested in DNA transfer, but the actual function of Tfs3 still remains widely unclear (Fernandez-Gonzalez and Backert, 2014). Some studies have suggested that this system constitutes a complete set of putative T4SS channels with other homologous genes (Mcclelland *et al.*, 2007). Alandiyjany *et al.* indicated a role for the *tfs3* T4SS in CtkA-mediated pro-inflammatory signalling by *H. pylori* (Alandiyjany *et al.*, 2017a). In addition, in the *tfs3* system of strain PeCan18B, homologs VirB4, VirB11, and VirD4 jointly form an enzyme complex related to the hydrolysis of adenosine triphosphate in the cytoplasmic membrane, providing sufficient energy for the transmembrane transport of macromolecular substrates (Fernandez-Gonzalez and Backert, 2014).

With the continual production of in-depth research on the pathogenic mechanism of *H. pylori*, the *tfs3* secretion system of *H. pylori* has attracted the attention of scholars. The discovery of the new transport system suggests the complexity, multiplicity, and strain-specificity of the pathogenic mechanism of *H. pylori*. This study investigated the role of VirD4 in the tfs3 gene cluster to examine the effect of VirD4 on gastric epithelial (GES) cells.

Results

VirD4 caused inflammation in GES-1 cells

First, GES-1 cells were treated with VirD4 at a concentration of 10 μg/mL for 0, 12, and 36 h. Under optical microscopy, we observed that over time, the cells appeared to shrink and swell gradually, varied in size, and exhibited obvious granulation, and the number of vacuoles and dead cells increased (Fig. 1A).

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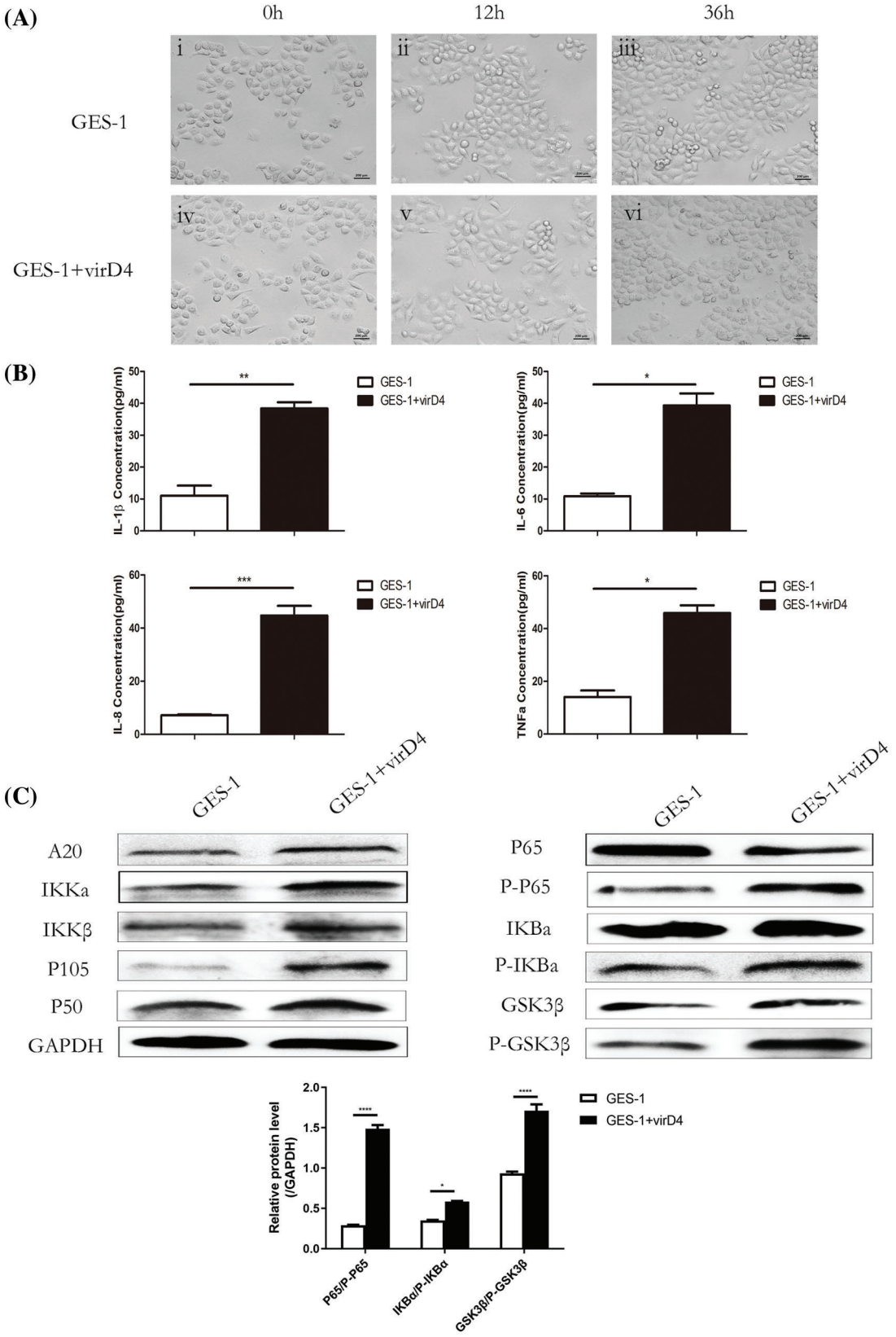

FIGURE 1. VirD4 affects the morphology of GES-1 cells and causes inflammation. (A) White light images were observed through phase-contrast microscopy after GES-1 cells were treated with VirD4 for 0, 12, and 36 h. Images in (i), (ii), and (iii) are of the control, and those in (iv), (v), and (vi) are of the treatment groups. Magnification, ×200; scale bar = 200 μm. (B) Quantitation of cytokine levels in culture supernatants of cells treated with VirD4 for 4 h using ELISA (IL-1β, IL-6, IL-8 and TNFα). (C) Expression of major proteins in the NF-kB pathway after the GES-1 cells were treated with VirD4 was examined using western blotting. Experiments were performed in triplicate with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.001$.

To explore whether VirD4 would alter the expression of inflammatory cytokines in GES-1 cells, the recombinant VirD4 protein was used to act on the cells *in vitro*. We then examined the effects of VirD4 on the secretion of proinflammatory cytokines in the GES-1 cells, after which the concentrations of these cytokines in cell culture supernatants were determined using an enzyme-linked immunosorbent assay. We observed that VirD4 increased the expression of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)α *in vitro* (Fig. 1B).

Signal transduction pathways are often activated in response to external stimuli. Nuclear factor (NF)-κB plays a vital role in *H. pylori* infection by regulating the expression of various inflammatory genes. Therefore, we investigated whether VirD4 would modulate the activity of NF-κB under our experimental conditions. We hypothesized that the increased expression of VirD4 promotes NF-kB pathway activation.

To test this hypothesis and further confirm the role of VirD4 in the regulation of inflammatory responses, proteins were extracted. We examined key molecules in the NF-κB pathway using western blotting; after the GES-1 cells were treated with VirD4, the expression of A20, IκB kinase (IKK)α, IKKβ, NF-κB P105, NF-κB P50, P65/phosphor (P)-P65, NF of IKBα/P-IKBα, and P-glycogen synthase kinase (GSK)3β/GSK3β were significantly induced, suggesting that VirD4 can accelerate NF-κB pathway activation (Fig. 1C). These results further corroborated our previous findings and suggested that VirD4 induced proinflammatory cytokine expression by increasing the activation of NF-κB in response to inflammation.

Role of VirD4 in the proliferation of GES cells

To examine the role of VirD4 in the proliferation of GES-1 cells, Cell Counting Kit-8 (CCK8) was used to detect the changes in proliferation of GES-1 cells under different concentrations of recombinant VirD4 proteins over time. The GES-1 cells exhibited good proliferation in the control group, but VirD4 inhibited the proliferative activity of the GES-1 cells (Fig. 2A).

Then, the colony formation assay was performed on GES-1 cells both with and without VirD4 treatment. VirD4 formed smaller clones than did the control cells (Figs. 2B and 2C). The results were consistent with those of CCK8.

The expression of a series of molecular markers was then detected in the GES-1 cells by using western blotting. As Fig. 2F shows, the expression of proliferating cell nuclear antigen, cyclinD1, and c-Myc considerably decreased in the GES-1 + VirD4 group. These results demonstrate that VirD4 expression affects GES-1 cell proliferation *in vitro*.

Effect of VirD4 on GES cell migration

Studies have demonstrated that *H. pylori* can alter migration ability in gastric cancer. To test whether VirD4 is responsible for the promotion of GES-1 cell migration, we performed a transwell migration assay, which demonstrated that the number of migrated cells after VirD4 treatment had remarkably increased compared with the control group (Figs. 2D and 2E).

The expression of a series of molecular markers was then detected in the GES-1 cells through western blotting. As shown in Fig. 2G, the expression of tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2 considerably decreased in the GES-1 + VirD4 group, whereas matrix metalloproteinases (MMP)1, MMP2, and MMP9 increased substantially compared with the control group. These results demonstrate that VirD4 expression affects GES-1 cell migration *in vitro*.

VirD4 caused apoptosis in GES-1 cells

To determine whether VirD4 affects GES-1 cell apoptosis, apoptotic cells were detected *in situ* by using annexin V-FITC/propidium iodide (PI) and Hoechst 33258 staining. First, GES-1 cells were stained with annexin V-FITC/PI after exposure to fixed concentrations of VirD4 for 4 h. The results revealed that after VirD4 treatment, intracellular green fluorescence increased (Fig. 3A). Next, we investigated apoptosis in the GES-1 cells through Hoechst 33258 staining. After the GES-1 cells were treated with VirD4, the number of cells with bright nuclear condensation or fragmented nuclei increased, which is characteristic of apoptosis (Fig. 3B).

Western blot analysis was also performed to analyze the changes in the apoptosis-promoting Bcl-2-associated X protein (BAX) and the apoptosis-inhibiting protein Bcl2 in cells from different treatment groups. Compared with the control group, the BAX index increased after treatment with VirD4, whereas the Bcl2 index decreased (Fig. 3C). These results indicated that the effect of GES-1 apoptosis induced in the GES-1 + VirD4 group was different from that of the control group, suggesting that VirD4 treatment in GES-1 cells promotes apoptosis and that the signaling molecules associated with apoptosis may change.

To further elucidate the role of VirD4 in the apoptosis of the GES-1 cell line, we detected the change in caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, and the substrates poly ADP-ribose polymerase (PARP) and cleaved PARP. Compared with the control cells, VirD4 reduced caspase-3 and caspase-9 and increased cleaved caspase-3 and cleaved caspase-9. In addition, VirD4 was able to cleave PARP because it decreased full-length PARP and increased c-terminal-cleaved PARP (Fig. 3D). Because the activation of the extracellular single-regulated kinase (ERK)1/2 pathway is also involved in positively regulating cell apoptosis, we detected the protein expression of P-ERK1/2 and Ras to evaluate whether it could also cause apoptosis in GES-1. The data indicated that the main proteins of the Ras/ERK signaling pathway remarkably decreased in the VirD4-treated GES-1 cells (Fig. 3E). These results demonstrate that VirD4 causes apoptosis in GES-1 cells partially by activating caspase and the ERK1/2/Ras pathway.

Discussion

H. pylori is a pathogenic bacterium that colonizes the human stomach, with a carrier rate of 50% in the global population. Its secretion transport system, T4SS, can contribute to various physiological and pathological reactions, such as inflammation, ulcers, and even cancer, caused by *H. pylori* through the transport of cell-related toxin CagA (Sue *et al.*, 2015; Yamaoka, 2010). *Tfs3* is a new pathogenic island similar to T4SS, and approximately one-fifth of all *H. pylori* strains carry the *tfs3* gene cluster (Alm *et al.*, 1999). Its function and pathogenic mechanism have garnered increasing attention. As one of the

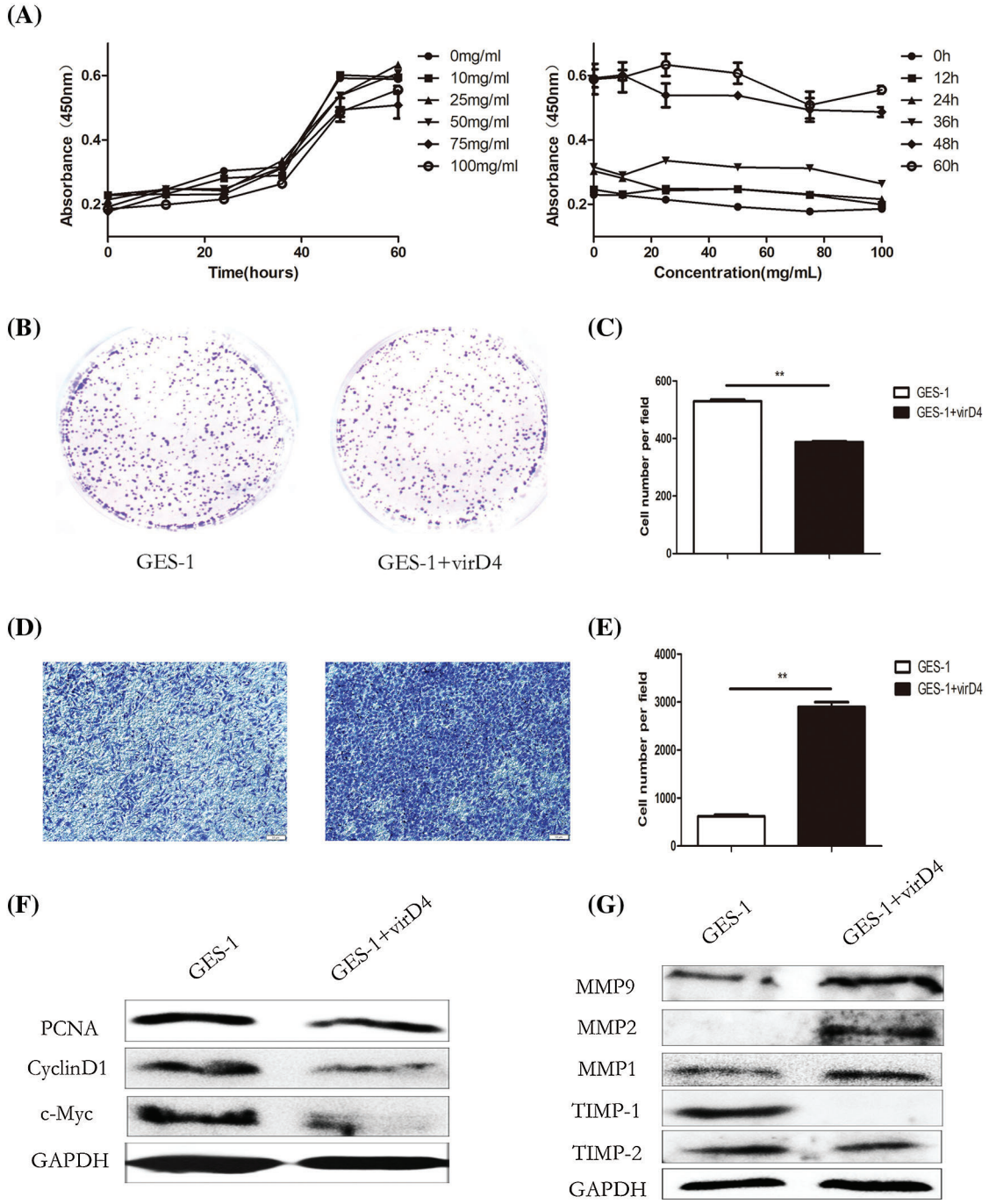

FIGURE 2. Effect of VirD4 on proliferation and migration of gastric epithelial (GES)-1. (A) Cell Counting Kit-8 assay performed on GES-1 cells indicated that VirD4 reduced cell proliferation in GES-1. (B–C) Colony formation assay performed on GES-1 cells indicated that VirD4 reduced cell proliferation. (D–E) Migratory ability of VirD4-treated GES-1 cells was evaluated using transwell migration assay. VirD4 caused the migration of the GES-1 cells. Magnification, ×100; scale bar = 10 μm. (F–G) Expression of the proliferation and migration related proteins was evaluated by immunoblotting after GES-1 cells were treated with VirD4 for 4 h. Experiments were performed in triplicate with similar results. $^{**}P < 0.01$.

fundamental components of *tfs3*, VirD4 plays a critical role in the assembly and transfer of CagA. Mutant strains lacking Cagβ, putative ATPases corresponding to VirD4 in prototypical T4SSs, were capable of T4SS core complex assembly but defective in CagA translocation into host cells (Lin *et al.*, 2020). However, few studies have described the function of VirD4 in *tfs3* secretion system. Therefore, identifying the function of VirD4 can deepen understanding of the pathogenic mechanism of the *tfs3* secretion system.

Studies have indicated that the upregulation of proinflammatory signals is a feature of certain *H. pylori* strains carrying *tfs3*, and many of the gene fragments are closely related to the expression and secretion of these inflammatory factors in GES cells (Alandiyjany *et al.*, 2017b; Kersulyte *et al.*, 2009). The present study demonstrated that GES-1 cells secrete small amounts of cytokines to maintain their physiological functions without stimulation, and after VirD4 treatment, the levels of proinflammatory cytokines

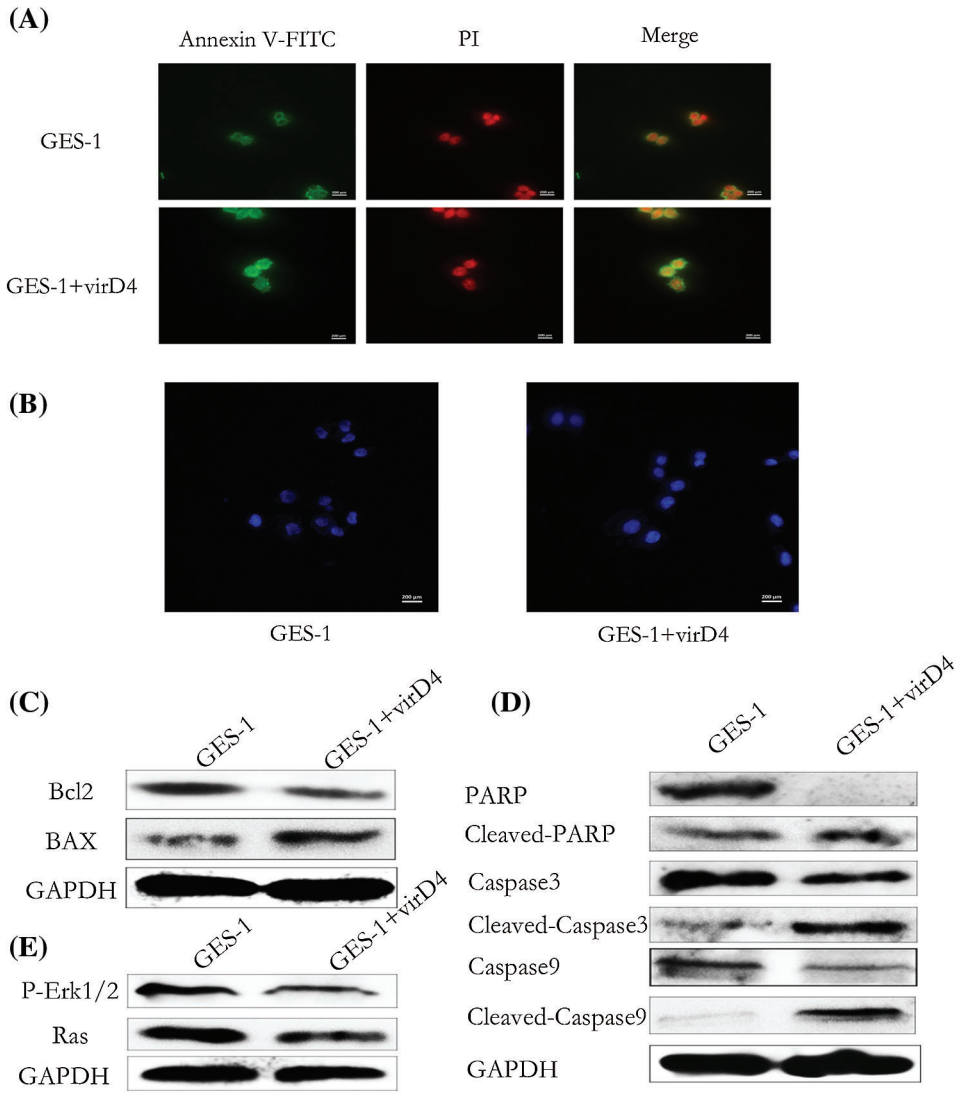

FIGURE 3. VirD4 promotes apoptosis and affects the expression of the apoptosis-Caspase family and the Ras/extracellular single-regulated kinase signaling pathways in GES-1 cells. (A) Annexin V-FITC/propidium iodide was used for situ observation through fluorescence microscopy of the apoptosis level of each group. Magnification, ×200; scale bar = 200 μm. (B) DNA segmentation of GES-1 cells detected by Hoechst 33258 stain. Magnification, ×200; scale bar = 200 μm. (C) Western blotting results suggested that apoptosis increased after GES-1 cells were treated with VirD4. (D–E) The level of proteins associated with the apoptosis-caspase family and the signaling pathway was evaluated using western blotting of GES-1. Experiments were performed in triplicate with similar results.

IL-1β, IL-6, IL-8, and TNFα increased, suggesting that the VirD4 protein can cause host cells to secrete inflammatory factors. Alandiyjany *et al.* (2017b) demonstrated that the activation of serine/threonine kinase on gastric mucosal cells depends on the T4SS encoded by the *tfs3* gene cluster to activate the NF-κB pathway and promote the release of inflammatory cytokines IL-8 and TNFα. Western blot was used to detect the major proteins of the NF-κB pathway in our study. The results indicated that the NF-κB pathway was activated after VirD4 acted on the GES-1 cells. Therefore, our results are consistent with those of other studies. VirD4 protein may play a role in potentiating *H. pylori*-mediated inflammatory responses in T4SS encoded by *tfs3*.

Studies have demonstrated that *H. pylori* can promote the proliferation and increase the migration of gastric mucosal epithelial cells (Alzahrani *et al.*, 2014; Zhu *et al.*, 2015). However, our results indicate that VirD4 proteins can inhibit the proliferation of GES-1 cells in a concentration-dependent and time-dependent manner; this result is consistent with the effect of VirD4 on the cell proliferation of human periodontal fibroblasts (Li and Liang, 2019). We also observed that VirD4 can cause a substantial migration of GES cells and that the expression of TIMP-1 and TIMP-2 decreased, whereas that of MMP1, MMP2, and MMP9 increased in the VirD4-treated cells. Ouyang *et al.* (2021) found that *H. pylori* infection caused epithelial-mesenchymal transition in GES-1, which in turn promoted the progression of gastric cancer. Our study shows an enhanced migration ability of gastric epithelial cells after *H. pylori* infection, which may be involved in the development and progression of *H. pylori* -associated gastric cancer. Therefore, the proliferation and migration in host cells changed by VirD4 in T4SS encoded by tfs3 may be involved in tumorigenesis.

Apoptosis is a process of cell contraction and autophagy, and it is one of the molecular mechanisms required to maintain homeostasis. It denotes the autonomous and

orderly death of normal cells after physiological and pathological stimulation (Fan *et al*., 2005). The most common apoptotic pathways are the death receptor pathway and the mitochondrial pathway (Ashkenazi *et al*., 2017; Czabotar *et al*., 2014). Studies have indicated that *H. pylori* can also cause the apoptosis of gastric mucosa epithelial cells (Lv *et al*., 2014). We used annexin V-FITC/PI and Hoechst 33258 staining to analyze the apoptosis of each group. We also detected protein expressions of BAX and Bcl2, which are crucial members of the apoptotic family (Bartchewsky *et al*., 2010). We discovered that VirD4 can cause apoptosis in GES-1 cells. Studies have demonstrated that apoptosis is strictly controlled by the caspase family and that the ERK signaling pathway is involved in the process (Tengku Din *et al*., 2018; Xu *et al*., 2019). To reveal the possible mechanism underlying the effect of VirD4 on GES-1 cells, we tested the main molecules in the caspase family and the Ras and ERK signaling pathways. We demonstrated that VirD4 can suppress the proteins of the Caspase family and reduce levels of P-ERK1/2 and Ras to promote apoptosis in GES-1 cells.

In conclusion, we investigated the effect of recombinant protein VirD4 of the *tfs3* secretion system on the inflammation, proliferation, migration, and apoptosis of GES-1 cells to elucidate the pathogenic mechanism of the *tfs3* secretion system. However, the crystal structure, subcellular location, composition, and function of this protein in the T4SS complex requires further investigation. Our study provides a new perspective for *H. pylori* -associated disease based on *tfs3* secretion system.

Materials and Methods

Recombinant VirD4 protein

The VirD4 gene segment was obtained through T-A cloning method. For this part, the DNA temple was obtained from the pylori clinical strain SBK which was cultured and maintained in our laboratory culturing on Columbia agar plates containing 10% sheep blood under microaerophilic conditions at 37°C. (The expression of VirD4 in different *H. pylori* type strains was showed in Suppl. Fig. S1.) Then the prokaryotic expression vector pET-28a (+)-VirD4 was constructed and transformed into *E. coli* Rosetta for the expression by induction of IPTG. The recombinant proteins were obtained and purified by KCL dyeing with gel cutting method, and identified via SDS-PAGE analysis.

Cell culture

The immortalized gastric epithelial mucosa cell line GES-1, established by the Beijing Institute for Cancer Research (Beijing, China) was cultured and maintained in our laboratory. The cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher, USA), supplemented with 10% fetal bovine serum (FBS; Life Technologies, USA) in a humidified atmosphere of 5% CO_2 at 37°C. For cell studies, 10 μg/mL purified recombinant VirD4 proteins were co-cultured with GES-1 cells for 4 h.

Western blot analysis

Cellular proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors on ice for 30 min. The cell lysates were centrifuged at 12,000 × *g* for 30 min, and protein contents were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Then, equal amounts of protein (200 μg) were resolved on 12% SDS-polyacrylamide gels by electrophoresis and transferred to PVDF membranes. The membranes were then incubated with 5% non-fat dry milk powder in tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature, before incubating them with primary antibody at 4°C overnight. After adding the appropriate secondary antibody (1:2000) at room temperature for 1 h, membranes were visualized using an enhanced chemiluminescence (ECL) system (Image Quant LAS 4000 mini, Pittsburgh, PA, USA) according to the instructions of the manufacturer. Target protein levels were normalized to GAPDH expression by performing densitometry, and relative fold-changes in protein levels were calculated; all experiments were repeated at least three times. Primary antibodies were as follows: GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA); Anti-A20, anti-IKKα, anti-IKKβ, anti-NF-κB P105, anti-NF-κB P50, anti-NF-κB P65, anti-P-NF-κB P65, anti-IKBα, anti-P-IKBα, anti-GSK3β, and anti-P-GSK3β, anti-PCNA, anti-CyclinD1, anti-c-Myc, anti-MMP1, anti-MMP2, anti-MMP9, anti-TIMP-1, anti-TIMP-2, anti-BAX, anti-Bcl2, anti-PARP, anti-cleaved-PARP, anti-Caspase3, anti-cleaved-Caspase3, anti-Caspase9, anti-cleaved-Caspase9, anti-P-Erk1/2, anti-Ras (1:500, all from Cell Signaling, Danvers, MA, USA); horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cytokine measurements

Particulates were removed from cell culture supernatants by centrifugation at 3000 × g for 10 min and the assay was immediately performed, or samples were aliquoted and stored at ≤ −20°C. Levels of active IL-6, IL-8, and TNF-a were measured using ELISA kits (eBioscience, Inc., San Diego, CA) following the instructions of the manufacturer.

Cell proliferation assay

The proliferation of GES-1 cell was examined using CCK-8 kit (Tongren, Shanghai, China) according to the manufacturer's instructions. Approximately 2×10^3 cells were seeded in 96-well plates and cultured for 24, 48, 72 and 96 h, respectively. 10 μL CCK-8 solution was then added to each well and incubated for 1 h. The absorbance at 450 nm was measured using automatic microplate reader (Bio-Rad, USA) at different time intervals.

Colony-formation assay

Cells were harvested and seeded into six-well plates (1000 cells/well) and incubated at 37°C in a 5% CO_2 humidified incubator for 10 days. The medium was changed at 3-day intervals. At the end of the incubation period, the cultures were fixed with 4% paraformaldehyde and stained with crystal violet.

Transwell migration assay

Transwell migration assay was performed using CoStar Transwell chambers (8 μm pore size; Corning, Costar, NY, USA). Cells (1×10^5/well) were seeded in the upper chambers of the wells in 200 μL serum-free medium, while the lower chambers were filled with 600 μL medium

containing 10% fetal bovine serum to induced cell migration. After incubation at 37°C in 5% CO_2 for 36 h, the cells in the upper surface of the membrane were removed with a cotton swab. Cells migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The images were obtained, and the cells were counted under a microscope.

In situ detection of apoptosis using V-FITC/PI staining

Cells at density of 2.5×10^5 were seeded on glass coverslips in 24-well dishes. After treatment time, the prepared apoptosis staining was added to each well according the manufacturer's protocol (Beyotime Biotechnology), removed the cover lips and place it on the slides, the slides were mounted and visualized using an inverted wide-filed fluorescence microscope (DeltaVision Elite, GE Healthcare Life Sciences, USA) to identify apoptosis.

Immunofluorescence analysis

Immunofluorescent staining was performed with cells grown on glass cover slips. Cultured cells were fixed for 30 min with 4% formaldehyde, then after rinsing in PBS, the cells were stained with Hoechst 33258 (Sigma, USA) for 5 min. The slides were mounted and visualized using an inverted wide-filed fluorescence microscope (DeltaVision Elite, GE Healthcare Life Sciences, USA).

Statistical Analysis

All results are presented as the mean ± SE of three independent experiments, and analysis was performed using SPSS 22.0 (SPSS, Chicago, IL, USA). Significant differences between groups were measured by performing a Student's *t*-test, and $P < 0.05$ was considered statistically significant. The experimental results are made by GraphPad Prism5.0.

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Availability of Data and Materials: All remaining data are available within the article, or available from the authors upon request.

Author Contribution: Conception and design: All authors; Administrative support: CZ; Provision of study materials or patients: CZ; Collection and assembly of data: YY, BY; Data analysis and interpretation: YY, BY; Manuscript writing: All authors; Final approval of manuscript: All authors.

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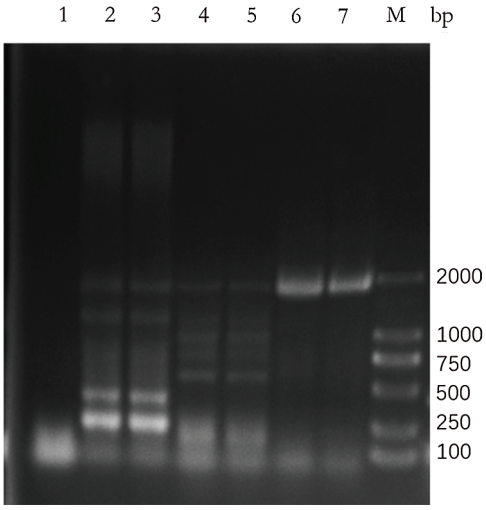

SUPPLEMENTARY FIGURE S1. Expression of VirD4 in different *H. pylori* type strains. The full length of VirD4 gene was about 1728 bp. VirD4 is highly expressed in the pylori clinical strain SBK (lanes 6, 7) but low expression in *H. pylori* type strain 11637 (lanes 2, 3) and 26695 (lanes 4, 5). Lane 1 is the negative control.