Genotoxic *Klebsiella pneumoniae* in Taiwan

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Abstract

**Background:** Colibactin is a nonribosomal peptide-polyketide synthesized by multi-enzyme complexes encoded by the *pks* gene cluster. Colibactin-producing *Escherichia coli* have been demonstrated to induce host DNA damage and promote colorectal cancer (CRC) development. In Taiwan, the occurrence of pyogenic liver abscess (PLA) has been suggested to correlate with an increasing risk of CRC, and *Klebsiella pneumoniae* is the predominant PLA pathogen in Taiwan.

**Methodology/Principal Findings:** At the *asr* tRNA loci of the newly sequenced *K. pneumoniae* 1084 genome, we identified a 208-kb genomic island, KPHP1208, of which a module identical to the *E. coli* *pks* colibactin gene cluster was recognized. KPHP1208 consists of eight modules, including the colibactin module and the modules predicted to be involved in integration, conjugation, yersiniabactin production, microcin production, and unknown functions. Transient infection of BALB/c normal liver cells with *K. pneumoniae* 1084 increased the phosphorylation of histone H2AX, indicating the induction of host DNA damage. Colibactin was required for the genotoxicity of *K. pneumoniae* 1084, as it was diminished by deletion of *clbA* gene and restored to the wild type level by trans-complementation with a *clbA* coding plasmid. Besides, BALB/c mice infected with *K. pneumoniae* 1084 exhibited enhanced DNA damage in the liver parenchymal cells when compared to the isogenic *clbA* deletion mutant. By PCR detection, the prevalence of *pks*-positive *K. pneumoniae* in Taiwan is 25.6%, which is higher than that reported in Europe (3.5%), and is significantly correlated with K1 type, which predominantly accounted for PLA in Taiwan.

**Conclusions:** Our knowledge regarding how bacteria contribute to carcinogenesis has just begun. The identification of genotoxic *K. pneumoniae* and its genetic components will facilitate future studies to elucidate the molecular basis underlying the link between *K. pneumoniae*, PLA, and CRC.

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Introduction

*Klebsiella pneumoniae* is a member of the family *Enterobacteriaceae*. The ability of *K. pneumoniae* to cause a wide range of human diseases, from urinary tract infections to life-threatening systemic infections, has attracted increasing attention to the pathogenesis of this bacterium [1–4]. In 1990s, *K. pneumoniae* surpassed *Escherichia coli* as the leading cause of community-acquired pyogenic liver abscess [5]. *K. pneumoniae*-caused liver abscess (KLA) was recognized by researchers in Taiwan and several areas since 1986. KLA is now considered an emerging disease worldwide [1–3,6]. Distinct from *E. coli*-caused liver abscess, KLA is genetically cryptogenic and is frequently complicated with metastatic lesions to other organs [7–9]. A significant proportion of KLA isolates in Taiwan belong to capsular serotype K1, with a prevalence rate ranging from 28% to 63% [10,11]. Several K1-specific genetic loci have been identified to contribute to *K. pneumoniae* virulence, including *magA* (for capsular polysaccharide synthesis), *kfu* (for iron uptake), and genes for the TonB-dependent iron acquisition system (*iucABCDiutA, archavinDCB*, and *yersiniabactin island*) [12–15].

Colorectal cancer (CRC) is a significant cause of morbidity and mortality throughout the world. Most CRC occur in people without a family history. Age, history of inflammatory bowel disease, and lifestyle are known risk factors for CRC. In Taiwan, CRC is the second most commonly diagnosed cancer. Its annual incidence increases steadily from 34.43/100,000 in year 2000 to 54.01/100,000 in year 2009 according to the report from the Department of Health in Taiwan. Pyogenic liver abscess has been suggested as a potential manifestation of CRC in Taiwan [16–19]. Intriguingly, an 11-year follow-up study in Taiwan demonstrated that patients with *K. pneumoniae*-caused PLA (KLA) were at increased risk of subsequent CRC than did patients with non*K. pneumoniae*-caused PLA [10].
pneumoniae PLA [20]. This finding raises a possibility that some bacterial virulence factors may endow K. pneumoniae with a cancer-inducing capacity.

Since the identification of an oncogenic role for Helicobacter pylori [21], our knowledge regarding how bacteria contribute to tumorigenesis has just begun. Acquisition of genomic instability is crucial for initiation and progression of tumors. Chronic exposure to DNA damaging agents may cause elevated genomic instability as a result of failure to repair damaged DNA or altered activation of the DNA damage-induced checkpoint responses [22]. Two bacterial products, cytotoxal distending toxin (CDT) [23] and colibactin [24] have been identified as genotoxins that can directly damage DNA, inhibit cell cycle, and trigger genomic instability in the host [22, 24]. CDT is a tripartite exotoxin consisting of CdtA, CdtB, and CdtC encoded by the cdtABC operon. Numerous bacteria have been found to produce CDT, including E. coli, H. pylori, Haemophilus ducreyi, Shigella dysenteriae, Salmonella enterica, and Campylobacter spp. [23, 25]. Colibactin, on the other hand, is a hybrid nonribosomal peptide-polykyde [24, 26] synthesized by multi-enzyme complexes that are encoded by genes carried on a 54-kb genomic region of E. coli [24]. The 54-kb chromosomal region, the pks colibactin gene cluster, was identified and fully sequenced in newborn meningitis E. coli strain HIE3034 [24]. In E. coli, the genotoxicity conferred by the pks colibactin gene cluster were demonstrated in vitro and in vivo [22, 24, 26]. By PCR, the presence of colibactin genes was detected in 53% of extraintestinal pathogenic E. coli (ExPEC) strains and in 34% of fecal E. coli isolates from healthy individuals [24]. The colibactin-positive E. coli isolates are almost exclusively classified in the phylogenetic lineage ECOR-B2 [24]. In addition to E. coli, few members of the family Enterobacteriaceae were found positive on the carriage of pks colibactin gene cluster, including Enterobacter aerogenes, Citrobacter koseri, and K. pneumoniae [27].

Horizontal gene transfer contributes to the evolution and emergence of pathogens by allowing virulence-associated genes to spread among bacteria [28]. Clusters of genes can be acquired as a unit known as genomic island. Several genomic islands have been discovered in K. pneumoniae, including the high pathogenicity genomic island HPI-ICEKp1 at the asn tRNA loci of K. pneumoniae NTUH K2044 [29], fimbral gene cluster-containing KPGI-5 at met tRNA loci of K. pneumoniae KR1116 genome [30], and several genomic islands related to carbohydrate metabolism and toxin synthesis [31, 32]. Recently, we determined the complete genome sequence of K. pneumoniae 1084 (GenBank Accession No. NC_018522.1) [33], which was a K1 strain isolated in Taiwan from a KLA patient. A comparative genomics approach was performed to identify potential determinants of virulence in the newly sequenced genome. Here we report the identification of a 208-kb chromosomal region with typical features of a genomic island at the asn tRNA loci of the K. pneumoniae 1084 genome. This 208-kb genomic island, named KPHP1208, is composed of 8 genomic modules (GMs). GM1, the first genomic module of KPHP1208, contains genes ~100% identical to those of the pks colibactin gene cluster reported in E. coli HIE3034 [24]. The colibactin-related genotoxicity of K. pneumoniae 1084 was validated by in vitro and in vivo experiments. The prevalence of colibactin genes among clinical K. pneumoniae isolates in Taiwan was also investigated and reported herein.

Materials and Methods

Ethics statement

Bacterial strains were isolated from patients with primary K. pneumoniae infections at Chung-Shan Medical University Hospital in central Taiwan during a 15-month period from April 2002. The isolates were cultured from clinical samples with no collection of patient identifiers or interaction with subjects. All animal experiments were performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Laboratory Animal Center (Taiwan), and the protocol was approved by the Animal Experimental Center of Chung-Shan Medical University.

Bacterial strains

A total of 207 non-repetitive K. pneumoniae isolates were collected in our previous study [11]. Among them, 35 (16.9%) were regarded as KLA strains as they were obtained from tissue-invasive cases that presented with the formation of liver abscesses, 59 (28.5%) were from cases associated with abscesses at non-hepatic sites, including lesions that occurred as empyema, endophthalmitis, necrotizing fasciitis, septic arthritis, along with lung, epideral, parotid, paraspinal, splenic, renal, prostate, muscle, and deep neck abscesses, and the remaining 113 (54.6%) isolates were obtained from non-abscess-related cases, including pneumonia without abscess, primary peritonitis, cellulitis, biliary tract infection, primary bacteremia with no original infectious foci identifiable, and catheter-related infections. The K. pneumoniae isolates were cultured in Luria-Bertani (LB) broth and stored at −80°C until use. Capsular antigens were determined by PCR detection of the K-serotype-specific wcx locus. Specific primers designed previously [7] were used to detect K1 type (GTA GGT ATT GGA AGC CAT GC and GCC CAG GTT AAT GAA TCC GT), and K2 type (GGA GCC ATT TGA ATT CGG TG and TCC CTA GGA CTT GCT TAA GT). K. pneumoniae 1084 is a K1 strain isolated from a diabetic patient with severe bacteremic liver abscess. Complete sequencing and annotation of K. pneumoniae 1084 was performed in our previous study (GenBank Accession No. NC_018522.1) [33].

Deletion and complementation of clbA

A 768-bp region spanning the coding sequence of clbA was deleted in K. pneumoniae 1084 by using an allelic exchange technique as described [34]. In general, ~1,200-bp DNA fragments flanking the region to be deleted were amplified with specific primer sets, p465 (AAA ATC TAG ACA TAG AGT TGG AGC AAC TGT T)/p466 (AAA AGG TAC CCT CAT TCC TGT TGA AAT GAG T), and p467 (AAA AGG TAC CTC TGA GCC GTC GAT AAT ATT GA)/p468 (AAA AGA GCT CTC CTA CCC TGG TAA TAT GCA GA) and the amplified DNA fragments were cloned into pKAS46, a suicide vector containing tpsL, which allows positive selection for vector loss using streptomycin [35]. After the occurrence of double crossover, the streptomycin-resistant but kanamycin-sensitive colonies were selected. The deletion of clbA was verified by PCR and Southern blot analysis and one of the confirmed mutants was named ΔclbA. For complementation experiments, a 735-bp DNA fragment containing full-length clbA gene was amplified using primers p178 (ATG AGG ATT GAT ATA TTA ATT GGA C) and p479 (ATT CTG CCC ATT TGA CGA ATG). The amplified DNA fragment was cloned into pCR®II (Invitrogen) to generate pYC502. Complementation of clbA was performed by introducing plasmid pYC502 into ΔclbA via electroporation.

In vitro infection assay

BALB/c normal liver (BNL) cells (ATCC TIB-73) were maintained by serial passage in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum at 37°C with 5% CO2. The BNL cells were synchronized by switched to serum
free medium for 16 hours. Approximately at 70% confluence, the BNL cells were washed four times with phosphate-buffered saline (PBS) and infected with *K. pneumoniae* 1084, ΔclbA, or ΔclbBFY502 at a multiplicity of infection (MOI) of 100 bacteria per cell. After a 4-h transient infection, BNL cells were washed with PBS and cultured with fresh DMEM supplemented with 5% fetal bovine serum (FBS) and 100 µg/ml gentamycin. For clonogenic assay, the control and infected cells were trypsinized to produce a single cell suspension. Equal amount of cells (1.5 × 10⁶) were seeded into a 35 mm culture dish. After incubation for 14 days, cells were fixed then stained with 0.5% crystal violet. The number of colonies for each group was counted under stereomicroscope in a double-blind manner. For Western blot, were counted for phospho-H2AX staining from images in a double-blind manner. To detect phospho-H2AX (γH2AX) foci in the nuclei, cells were fixed with 95% methanol-5% acetic acid, blocked with PBS-0.1% Tween 20–2% skim milk for 30 min, probed with rabbit anti-phospho-H2AX antibody (Cell Signaling Technology), and then hybridized with goat anti-rabbit-Alexa488 antibodies (Invitrogen). A Zeiss LSM510 CLSM (Carl Zeiss) with a 60× oil objective was used for imaging. At least 300 cells per group were counted for phospho-H2AX staining from images in a double-blind manner. For Western blot, ~10⁷ cells were collected and lysed with 1000 µl of lysis buffer (PBS, 1% Triton X-100, with protease and phosphatase inhibitors). After denaturation by boiling for 5 min with loading buffer, 30 µg of total proteins were separated on 12% of SDS gel, transferred to PVDF membrane (BioRad), blocked with 2% skim milk, and probed with anti-phospho-H2AX (Cell Signaling Technology), or with anti-Histone H3 (Cell Signaling Technology) antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence detection.

**In vivo infection assay**

Male BALB/c mice were purchased from the National laboratory animal center (NLAC Taiwan) at 7 wk of age and allowed to acclimatize in the animal house of Chung Shan Medical University for 1–2 wk before experiments. Mice were starved for 16 h prior to infection. PBS or 100 µl of bacterial suspension containing 10⁷ CFU of *K. pneumoniae* 1084, ΔclbA, or ΔclbBFY502 was respectively inoculated into groups of mice (6 mice a group) via an oral route as described in our previous study [34]. The PBS-inoculated or *K. pneumoniae*-infected mice were sacrificed at 1 day or 2 days post-inoculation. The liver was recovered immediately. The retrieved liver was divided into two parts, one was homogenized for determination of bacterial loads and detection of the level of phospho-H2AX by Western blot analyses, and the other was fixed in 4% paraformaldehyde at 4°C for 24 hours then embedded in paraffin. After deparaffinization and rehydration, slides were prepared and incubated with boiling sodium citrate buffer (pH 6.0; 10 mM) for 10 min to unmask antigens, and stained with rabbit anti-phospho-H2AX (Thermo Fisher Scientific) antibodies. Signals were detected by using a NovoLink Polymer Detection System (Leica Biosystem). Counterstaining was done with nuclear Hematoxylin. Images were acquired with a Nikon light microscope. Three sections were randomly selected from each of the samples retrieved from experimental mice in a double-blind manner. At least 1000 hepatocytes per section were counted for phospho-H2AX-positive staining.

**PCR detection of pks colibactin genes**

The presence of pks colibactin genes among clinical *K. pneumoniae* isolated in Taiwan was determined by PCR with primers as published in a previous study [24]. Briefly, primers for clbB and clbV were clbBF (GAT TTG GAT ACT GGG GAC AAC CG), clbBR (CTT CCC GTT TGA GCA CAC), clbNF (GTT TTG TTC CTC GCC AGA TAG TCA TTC), and clbNR (CAG TTT GGG TAT G1G TGG AAG G). Primers for two internal loci within the pks colibactin gene cluster, clbA (phosphophantetheinyl transferase) and clbQ (diol-sterase), were clbAF (CTA GAT GAT CCG TGG CGA TTG), clbAR (CAG ATA CAC AGA TAC CAT TCA), clbQF (CTT GTA TAG TTA CAC AAC TAT TCA), and clbQR (TAA TCC TGT TAG CTT TCG TTC). Genomic DNA (gDNA) samples for the 207 *K. pneumoniae* strains were prepared as described [11]. An initial denaturation at 95°C for 10 min was followed by denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 1 min for 30 cycles. *K. pneumoniae* 1084 was used as the positive control and *K. pneumoniae* CG43 was used as the negative control for the PCR experiments.

**Statistical analysis**

The Mann-Whitney U test, Student’s t test, or Fisher’s exact test were used to determine differences between groups suggested. Statistical significance was determined based on a two-tailed P value <0.05.

**Results and Discussion**

**Genetic structure and comparative analysis of KPHPI208**

At the *asn* tRNA loci, an integration hotspot for foreign mobile DNA elements, we identified a 208-kb region exhibiting typical features of a genomic island and named it KPHPI208. A total of 135 protein-coding genes were contained in this island. Its overall G+C% is 49.6, which is lower than that of the entire genome of *K. pneumoniae* 1084 (57.4%). Comparative genomics analysis of this region had identified eight genomic modules (GM1–GM8) each displaying a conserved genetic organization and were likely exchangeable among bacteria genomes. As shown in Figure 1, KPHPI208 accommodates a pks colibactin module (GM1), 5 modules (GM2, GM3, GM4, GM5, and GM7) similar to those contained within HPI-ICE*Kp1* [29] and HPI-ICE*El1* [36], a microcin module (GM6), and a novel module (GM8). The insertion site of KPHPI208 is located at the *asn* tRNA locus near the *ompS* gene of the *K. pneumoniae* 1084 genome. Homologous regions of GM2, GM3, GM4, and GM5 were identified in the genome of *Enterobacter hormaechei* 05-545 spanning the high pathogenicity island HPI-ICE*El1* [36], and in the genome of *Escherichia coli* ECOR31 at the integrative and conjugative element ICE*El1* [37]. GM2 is an 18-kb module containing genes for integration and conjugation. GM3 is a 30-kb module carrying genes for yersiniabactin production. GM4 and GM5 harbor genes of unknown functions (Figure 1). Homologues of GM2, GM3, GM5, and GM7 also present in the 76-kb high pathogenicity island HPI-ICE*Kp1* of *K. pneumoniae* NTUH K2044 [29]. The insertion site of HPI-ICE*Kp1* in the genome of NTUH K2044 is also the *asn* tRNA locus near *ompS*. The nucleotide sequence of GM6 is ~100% identical to the microcin E492 gene cluster of *E. coli* [32]. The 22-kb region consisting of genes for the production of microcin and immunity protein locates next to the second *asn* tRNA locus in KPHPI208. The eight modules (GM1-GM8) of KPHPI208 are bounded by 4 *asn* tRNA genes, 8 *attO* sites, and 4 *intB* integrase genes (Figure 1). The *attO* is a conserved 17-bp direct repeat that served as an integration site for integrative and conjugative elements (ICEs). In compared to HPI-ICE*Kp1*, which has only one integrase gene (*intB1) in the yersiniabactin module, KPHPI208 has three additional *intB* genes, *intB2, intB3*, and *intB4*, carried by GM4, GM6, and GM8, respectively (Figure 1). Acquisition of the three modules flanked...
by attO and asn tRNA was probably the result of insertion events occurred at the three asn tRNA loci of the \textit{K. pneumoniae} genomes (Figure 1).

GM1, the largest module in KPHP208, consists of genes responsible for colibactin production. Nucleotide sequence of GM1 is \textasciitilde100\% identical to that of the \textit{pkb} colibactin gene cluster reported in \textit{E. coli} IHE3054 [24] (Figure 2). Colibactin is a peptide-polyketide hybrid compound. For the synthesis of colibactin, the \textit{pkb} colibactin gene cluster encodes several proteins including non-ribosomal peptide megasynthases (NRPS), polypeptide megasynthases (PKS), hybrid NPRS/PKS megasynthases, and accessory, tailoring, and editing enzymes [24]. GM1 of KPHP208 accommodates all of the \textit{pkb} genes required for the synthesis of colibactin (Figure 2). In the genome of \textit{E. coli} IHE3054, the \textit{pkb} colibactin gene cluster is flanked by attO direct repeats at both sides, and an additional P4-like integrase gene, \textit{intP4}, is located next to the end of the gene cluster near \textit{clbO} [27]. Although the \textit{pkb} colibactin gene cluster carried in KPHP208-GM1 is identical to the \textit{E. coli} version, the \textit{intP4}, together with one of the attO next to it, are not found. This is reminiscent of a previous study, in which five \textit{K. pneumoniae} isolates from Europe were PCR-detected positive on the presence of colibactin genes. In the study the \textit{intP4} associated with the colibactin gene cluster was reported to be missing in \textit{K. pneumoniae} [27]. Sequencing of KPHP208 not only confirmed this genetic structure but also uncovered the complete genetic structure of the genomic island by discovering additional genomic modules in this region. In addition, the variable-number tandem repeat (VNTR) between \textit{clbB} and \textit{clbR} of KPHP208-GM1 has 16 ACAGATAC repeats. It repeats in \textit{E. coli} IHE3054 (Figure 2).

Genotoxicity of \textit{K. pneumoniae} 1084

Exposure to colibactin-producing \textit{E. coli} was demonstrated to inflict double strand breaks (DSBs) of host DNA in vitro and in vivo [22,24,26]. Discovery of the \textit{pkb} colibactin gene cluster in KPHP208-GM1 (Figure 2) encouraged us to examine whether this gene cluster endowed \textit{K. pneumoniae} 1084 with genotoxicity. DSBs of host DNA induce phosphorylation of the histone protein H2A variant (H2AX) at serine 139 that generates \(\gamma\)-H2AX through the activation of ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3-related protein) kinases. Accumulation and retention of \(\gamma\)-H2AX can therefore serve as an indicator for the signaling cascade induced by DNA damage [38]. In this study, BALB/c normal liver (BNL) cells were used as a cell model to examine the degree of DNA damage induced by \textit{K. pneumoniae} 1084 infection. Compared to the uninfected control (Figure 3A), a number of \(\gamma\)-H2AX foci were noted in the nuclei of \textit{K. pneumoniae} 1084-infected BNL cells at 4 h post-infection (green fluorescent signals in Figure 3B). Because the \(\gamma\)-H2AX foci on mitotic chromosomes represent unrepaired DNA breaks or scar of repaired lesions [39,40], this result suggests an induction of DNA damage by \textit{K. pneumoniae} 1084 infection. A phosphatethionetyl transferase is encoded by \textit{clbA} that is essential for the synthesis of colibactin [24]. It was demonstrated in \textit{E. coli} that the genotoxicity in host cells was significantly attenuated by the loss of \textit{clbA} [22,26]. To verify whether the \textit{K. pneumoniae} 1084-induced DNA damage was attributed by colibactin, we deleted a 769-bp region spanning the coding sequence of \textit{clbA} (Figure 2) in \textit{K. pneumoniae} 1084 to generate an isogenic \textit{clbA} mutant and named it \(\Delta\textit{clbA}\). Similarly, the \(\gamma\)-H2AX foci were hardly detected in the \textit{K. pneumoniae} \(\Delta\textit{clbA}\)-infected BNL cells (Figure 3C). By Western blot analyses, time-dependent accumulation of \(\gamma\)-H2AX was noted in the transiently infected BNL cells, which were recovered at 2, 4, and 6 h after a 4-h infection with \textit{K. pneumoniae} 1084 (Figure 3F), as compared with the uninfected control (Figure 3G). The \(\gamma\)-H2AX signal was diminished in the \(\Delta\textit{clbA}\)-infected group (Figure 3F). By introduction of the \textit{clbA}-complementing plasmid pYC502, the reduced genotoxicity of \(\Delta\textit{clbA}\) was restored to the wild type level (Figure 3D, 3E and 3F). The result demonstrated the genotoxicity of \textit{K. pneumoniae} 1084, which was conferred by the colibactin genes accommodated in KPHP208-GM1. Viability of the \textit{K. pneumoniae} 1084-infected cells was unaffected upon a 4-h transient infection. To demonstrate the consequence of the DNA damage induced on BNL cells by infection with \textit{K. pneumoniae} 1084, we performed a conventional clonogenic assay. After 14-day incubation, the number of colonies formed in the \textit{K. pneumoniae} 1084- and the complement strain (\textit{K. pneumoniae} 1084-pYC502)-infected group was significantly greater than that in the \(\Delta\textit{clbA}\)-infected group (Figure 3H and 3I). This result suggested that the accumulation of \textit{K. pneumoniae} 1084-induced DNA damage might contribute to tumorigenesis.

To further determine whether \textit{K. pneumoniae} 1084 elicited DNA damage in vivo, \(10^7\) CFU of \textit{K. pneumoniae} 1084 were orally inoculated into 8–10 week old BALB/c mice. At 1-day or 2-day post-inoculation, mice were sacrificed and the liver was retrieved for tissue section. Compared with the PBS-inoculated control mice (Figure 4A), extensive distribution of \(\gamma\)-H2AX foci were observed in the nuclei of liver parenchymal cells infected with \textit{K. pneumoniae} 1084 (Figure 4B) or with the complement strain (\(\Delta\textit{clbA}\)-pYC502) (Figure 4C). The liver sections were further stained with hematoxylin to demonstrate the extent of liver inflammation. The majority of liver damages were observed in the \(\Delta\textit{clbA}\)-infected group (Figure 4C). The results indicated that this strain expressed a high level of genotoxicity in vivo. This, to our knowledge, is the first demonstration of the genotoxicity in vivo of a \textit{K. pneumoniae} strain.
The regions spanning the genes responsible for colibactin production were depicted as arrows according to the directions of transcription. The attO sites in the left were marked by solid triangles. The VNTR locus between clbB and clbR was marked by empty triangles. The 53-kb regions indicated by dotted lines are ~100% identical. The locations of the four PCR amplicons in studying the prevalence of the colibactin genes among K. pneumoniae clinical isolates were marked. The 768-bp region spanning the clbA gene, which was deleted in ΔclbA strain, was indicated.

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Figure 2. The pks colibactin gene cluster (GM1) in KPHPI208 and the pks colibactin gene cluster in the E. coli IHE3034 genome. The prevalence of the colibactin genes among clinical K. pneumoniae isolates in Taiwan

The presence of pks colibactin gene cluster as a part of the mobilome suggests its potential to spread among local K. pneumoniae strains. To investigate the prevalence of colibactin genes (Figure 2) among K. pneumoniae isolates in Taiwan, PCR detection was carried out on a collection of 207 clinical strains obtained from patients with primary K. pneumoniae infections. The pks colibactin gene cluster markers clbB and clbN [41] were jointly detected in 53 (25.6%) of the 207 K. pneumoniae strains. PCR detection of two additional colibactin genes clbA and clbQ yielded results concordant with those for clbB and clbN. Approximately 66% (35/53) of the colibactin-positive K. pneumoniae were the K1 type (Table 1). Statistical analysis revealed a significant correlation between the carriage of colibactin genes and K1 type (odds ratio, 19.4; 95% confidence interval, 8.8–42.9; p<0.0001). However, the presence of colibactin genes appeared to be irrelevant to the type of infection, as the colibactin-positive K. pneumoniae distributed evenly among isolates collected from cases of KLA (8/35; 22%), non-hepatic abscess (17/59; 28%), and non-abscess infections (28/113; 25%). The overall colibactin-positive rate was 25.6% (53/207). This was significantly higher than that reported by Putze et al, in which only five out of 141 (3.5%) K. pneumoniae strains isolated from Europe were colibactin-positive [27]. Our result indicates a close relatedness of the colibactin gene cluster to K1 type. The relatively higher prevalence of colibactin genes demonstrated here might be related to the fact that serotype K1 was the most frequently identified type in Taiwan [42], but it ranked eighth in European survey [43].

Chronic inflammation has been recognized as a risk factor for CRC and other cancers. In a colitis-associated CRC mouse model, the proliferation of a genotoxic E. coli commensal strain, NC101, was demonstrated to be favored by an inflammatory milieu and thus promoted tumorigenesis of CRC [26]. Several clinical studies have demonstrated the relationship between CRC, PLA and K. pneumoniae in Taiwan [17,18,20]. The hazard ratio of CRC was 3.36 times greater for patients with PLA as reported in a nationwide population-based 5-year follow-up study comprised of 274 PLA patients and 1,370 randomly selected subjects [18]. In another nationwide cohort study in Taiwan, of which 1257 PLA patients without prior cancers were followed-up from 2008–2012, it was reported that the incidence of CRC in PLA patients was significantly raised as compared to the control group [17]. In addition, a retrospective study of PLA patients at a medical center in Taiwan has further indicated that the risk of CRC was 2.68 times greater for patients with K. pneumoniae-caused PLA than those with non-K. pneumoniae PLA [20]. Our discovery on the high prevalence of colibactin-positive K. pneumoniae in Taiwan, predominating among K1 strains, therefore poses an intriguing opportunity in studying the link between genotoxic K. pneumoniae and...
CRC. Whether the CRC-associated KLA strains correlate to the presence of colibactin genes remains to be elucidated. However, as we report here that 25–28% of K. pneumoniae isolates from non-KLA infections were also colibactin-positive, the possibility that other types of K. pneumoniae infections may contribute to the development of CRC and/or other types of cancers cannot be ignored. The role of genotoxic K. pneumoniae in tumorigenesis deserves further studies.

**Figure 3.** K. pneumoniae 1084 induced colibactin-related DSBs *in vitro*. BNL cells were left uninfected (A) or were infected with K. pneumoniae 1084 (B), ΔclbA (C), or with ΔclbA complemented with clbA coding plasmid pYC502 (D) at an MOI of 100. After 4 h infection, the cells were washed, co-cultured with gentamycin (100 μg/ml), and were examined by confocal microscopy for DNA (blue, stained with Hoechst 33342), for membrane glycoproteins (red, stained with ConA), and for γH2AX (green, recognized by Alexa488-anti-γH2AX antibodies) (scale bar, 20 μm). (E) Quantification of γH2AX-positive cells. Error bars represent SEs from three experiments. (F) Western blot analyses of γH2AX or H3 in BNL cells recovered at 2, 4, and 6 h after a 4 h transient infection with K. pneumoniae 1084 (lanes 1–3), ΔclbA (lanes 4–6), or with ΔclbA complemented with clbA-coding plasmid pYC502 (lanes 7–9). (G) Western blot analyses of γH2AX and H3 in uninfected BNL cells harvested from serum recovery for 2, 4, 6, 24, 48, and 72 h. (H) Clonogenic assays. BNL cells were uninfected (Control) or transiently infected with K. pneumoniae 1084, ΔclbA, or with ΔclbA-pYC502 for 4 hours. Colonies formed after 14-day incubation stained with 0.5% of crystal violet. A representative image is presented. (I) Quantification of colony formation. Error bars represent SEMs from three experiments. An asterisk (*) represents a significant increase in the K. pneumoniae-infected group in comparison with the uninfected control by the Student’s *t* test (two-tailed; *P* < 0.05). doi:10.1371/journal.pone.0096292.g003
Conclusions

Here we report the complete genetic structure of KPHPI208 analyzed by comparative genomics approaches. The 208-kb genomic island contains eight genomic modules (GM1-GM8), including a \( pks \) colibactin gene cluster which was first identified and characterized in \( E. coli \) IHE3034 [24]. The \( pks \) colibactin gene cluster within the GM1 of KPHPI208 shares a striking \( \sim 100\% \) sequence identity with the \( E. coli \) version, suggesting that their functions and regulation are conserved. Accumulating evidence has pointed out the role of colibactin-producing \( E. coli \) in the development of chronic inflammation and CRC in humans.

Figure 4. DNA damage evoked by \( K. pneumoniae \) 1084 infection \textit{in vivo}. For all the experimental groups, liver sections were prepared from the liver retrieved at 2-day post-inoculation, stained with anti-\( \gamma\)-H2AX antibodies, and imaged under microscopic observation with magnification of \( \times 400 \). Representative liver section of the PBS-inoculated control mice (A), the \( K. pneumoniae \) 1084-infected mice (B), the \( \Delta clbA \)-infected mice (C), and the \( \Delta clbA \)-pYCS02-infected mice (D) are shown. Scale bar: 50 \( \mu m \). (E) Quantification of \( \gamma\)-H2AX-positive hepatocytes. Each value is the mean \( \pm \) SEM for 3 mice. An asterisk (*) represents a significant decrease in the \( \Delta clbA \)-infected group in comparison with the \( K. pneumoniae \) 1084-infected group by the Mann-Whitney U test (two-tailed; \( P<0.05 \)). (F) Bacterial loads of the liver determined at 1-day and 2-day infection with \( K. pneumoniae \) 1084 (black bars), \( \Delta clbA \) (white bars), or \( \Delta clbA \)-pYCS02 (slash bars). Ten-folded dilutes of liver homogenates were plated onto M9 agar for enumerating CFU of \( K. pneumoniae \). Each value is presented as the average bacterial loads \( \pm \) SEM (Log CFU/g) for 3 mice a time point. Statistical analysis by the Mann-Whitney U test showed no significant difference between the \( K. pneumoniae \) 1084-infected and the \( \Delta clbA \)-infected groups. (G) Liver lysates were prepared from PBS-control, \( K. pneumoniae \) 1084-, \( \Delta clbA \), and \( \Delta clbA \)-pYCS02-infected mice at 1-day and 2-day post-inoculation. The sample size in each experimental group for each time point is 3. Eighty micrograms of total proteins were subjected to Western blot analyses with specific antibodies against \( \gamma\)-H2AX and H3. A representative result from at least three experiments is shown. (H) Band intensity of \( \gamma\)-H2AX and H3 was determined by Densitometry calculation and the average ratio of \( \gamma\)-H2AX to H3 is presented. Each value is the mean \( \pm \) SEM for 3 mice. An asterisk (*) represents a significant decrease in the \( \Delta clbA \)-infected group in comparison with the \( K. pneumoniae \) 1084-infected group by the Mann-Whitney U test (two-tailed; \( P<0.05 \)).

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Table 1. Factors associated with colibactin-positive *K. pneumoniae* isolates in Taiwan.

| K type* | K1 (n = 49) | K2 (n = 35) | Clinical characteristics | K1 (n = 49) | K2 (n = 35) | KLA* (n = 59) | Non-hepatic abscess* (n = 59) | Non-abscess* (n = 113) |
|---------|-------------|-------------|--------------------------|-------------|-------------|-------------|-----------------------------|----------------------|
| colibactin (n = 53)* | 35 (71) | 9 (26) | Pyogenic infection (n = 94) | 8 (22) | 17 (28) | 28 (25) |                           |                      |
| OR (95% CI) | 19.4 (8.8–42.9) | 1.0 (0.5–2.0) | Non-abscess* (n = 113) | 0.8 (0.4–2.0) | 1.3 (0.6–2.5) | 0.9 (0.5–1.7) |                      |                      |
| p-value | <0.0001 | 1 |                           | 0.8325 | 0.5969 | 0.8731 |                      |                      |

Data are no. (%) of isolates. Statistical comparisons by Fisher’s exact test are between the pks-positive group and the pks-negative group.

* K. pneumoniae strains in which the clbB and clbN were simultaneously detected by PCR with gene-specific primers are considered colibactin-positive.

1. K. pneumoniae strains from tissue-invasive cases that presented with the formation of liver abscesses were regarded as KLA isolates.

2. K. pneumoniae strains from cases associated with abscesses at non-hepatic sites, including lesions that occurred as empyema, endophthalmitis, necrotizing fasciitis, septic arthritis, along with lung, epidural, parotid, paraspinal, splenic, renal, prostate, muscle, and deep neck abscesses.

The discovery of genotoxic *K. pneumoniae* as well as the determination of complete genetic environment will help to elucidate the link between *K. pneumoniae*, PLA, and CRC.

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Author Contributions

Conceived and designed the experiments: YCL MKC CYL YTC. Performed the experiments: YCL ACL YHD CCH MCL YTC. Analyzed the data: YCL YTC. Contributed reagents/materials/analysis tools: YCL MCL YTC. Wrote the paper: YCL YTC.
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