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Ceftazidime-avibactam resistance in KPC-producing Klebsiella *pneumoniae* accompanied hypermucoviscosity acquisition

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Abstract

Background Antimicrobial resistance and bacterial hypermucoviscosity, associated with escalating production of capsules, constitute major challenges for the clinical management of Klebsiella *pneumoniae* (*K. pneumoniae*) infections. This study investigates the association and underlying mechanism between ceftazidime-avibactam (CAZ-AVI) resistance and bacterial hypermucoviscosity in Klebsiella *pneumoniae* carbapenemase (KPC)-producing *K. pneumoniae* (KPC-Kp).

Results The proportion of CAZ-AVI-sensitive clinical isolates exhibiting the hypermucoviscous phenotype was significantly lower than that of the resistant strains (5.6% vs. 46.7%, $P < 0.001$). To further verify the correlation and molecular mechanism between CAZ-AVI resistance and hypermucoviscosity, 10 CAZ-AVI-resistant isolates were generated through in vitro resistance selection from CAZ-AVI-sensitive KPC-Kp. The results showed the same association as it showed in the clinical isolates, with four out of ten induced CAZ-AVI-resistant isolates transitioning from negative to positive in the string tests. Comparative genomic analysis identified diverse mutations in the *wzc* gene, crucial for capsule polysaccharide (CPS) synthesis, in all four CAZ-AVI-resistant hypermucoviscous KPC-Kp strains compared to the parent strains. However, these mutations were absent in the other six KPC-Kp strains that did not exhibit induced hypermucoviscosity. Cloning of the *wzc* gene variants and their expression in wild-type strains confirmed that mutations in the *wzc* gene can induce bacterial hypermucoviscosity and heightened virulence, however, they do not confer resistance to CAZ-AVI.

Conclusions These results indicated that resistance to CAZ-AVI in KPC-Kp isolates may be accompanied by the acquisition of hypermucoviscosity, with mutations in the *wzc* gene often involving in this process.

Keywords Ceftazidime-avibactam resistance, KPC, Hypermucoviscosity, *wzc*, *K. pneumoniae*

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Introduction

Carbapenem-resistant Klebsiella *pneumoniae* (*K. pneumoniae*) (CRKP) is now becoming a global concern due to its resistance to carbapenem antibiotics, posing a significant challenge to the effectiveness of carbapenem therapies, which are considered to be the last line against antimicrobial resistance [1–3]. Carbapenemases categorized into class A [Klebsiella *pneumoniae* carbapenemase (KPC)], class B [New Deli metallo-beta-lactamase (NDM), Imipenemase (IMP), and Verona integron-encoded metallo-beta-lactamase (VIM)], and class D [Oxacillinase (OXA)-48-like], are the primary drivers of CRKP resistance to nearly all antibiotics [4]. In China and other countries, the spread of CRKP is largely attributed to the prevalence of KPCs produced by over 70% of CRKP strains [5, 6].

Avibactam (AVI) is a novel beta-lactamase inhibitor that can inactivate class A, class C, and certain class D beta-lactamases [7]. The effectiveness of ceftazidime-avibactam (CAZ-AVI) in restoring susceptibility to ceftazidime in over 80% of KPC-producing *K. pneumoniae* (KPC-Kp) strains has already been demonstrated [8–10]. Consequently, CAZ-AVI has become an indispensable treatment option when KPC-Kp exhibits resistance to most available antibiotics. Recently, the Infectious Diseases Society of America has identified CAZ-AVI as one of the primary choices for treating KPC-Kp infections, with the exception of the urinary tract infections [11]. However, the emergence of CAZ-AVI resistance in KPC-Kp has been reported recently [12–14]. Resistance to CAZ-AVI predominantly stems from mutations in the Ω-loop of KPC, which are considered to hinder the binding of AVI [12, 14]. Additionally, mutations in PBP3 (encoded by *ftsI*) and some certain porins such as OmpK35/OmpK36 also contribute to CAZ-AVI resistance in KPC-Kp strains [7, 15].

Hypermucoviscous *K. pneumoniae* (HMKP) produces an abundance of capsule and exhibits a hypermucoviscous phenotype when cultivated on agar plates. HMKP is associated with various invasive infections, such as pyogenic liver abscesses, and is commonly regarded as hyper-virulent [16, 17]. The hypermucoviscous phenotype is related to the expression of genes involved in capsule polysaccharide (CPS) synthesis, including *wzi*, *wzc* and *wzy*, which are regulated by the regulator of mucoid phenotype A (*rmpA*) [18–21]. Capsules can prevent cell adherence and phagocytosis, thereby enhancing virulence. Furthermore, the loss of the hypermucoviscous phenotype is associated with reduced virulence [18, 22–24]. The emergence of carbapenem-resistant HMKP (CR-HMKP) has attracted attention recently [25]. However, currently, no comprehensive investigation has been reported regarding the correlation between CAZ-AVI resistance and hypermucoviscosity in *K. pneumoniae*.

In this study, we aimed to explore the correlation between CAZ-AVI resistance and the hypermucoviscous phenotype by clinically collecting both CAZ-AVI-sensitive and -resistant KPC-Kp isolates and confirming the presence of the bacterial hypermucoviscosity. Additionally, the association and its underlying mechanism were validated through in vitro multipassage resistance selection.

Methods

Strains and antimicrobial susceptibility testing

All clinically isolated KPC-Kp strains from inpatients included in this study were retrospectively collected from strains previously stored in the clinical diagnostic department of hospitals in Guangdong, China from 2018 to 2020. Duplicate strains isolated from the same patient were excluded from the analysis. Selection of strains for this study was randomized based on the molecular prevalence characteristics of KPC-Kp. The identification of *K. pneumoniae* isolates was conducted using the VITEK-2 automated platform (bioMérieux, Marcy l'Etoile, France). The presence of bla_{KPC} was confirmed using polymerase chain reaction (PCR), with the specific primers listed in Table S1.

Additionally, PCR was conducted to identify bla_{KPC} mutations in CAZ-AVI-resistant KPC-Kp strains and *wzc* mutations in hypermucoviscous phenotype strains. To determine the capsular serotypes, PCR was performed to amplify the *wzi* gene in all KPC-Kp strains, following a previously described protocol [26]. All PCR products were sequenced by Sanger sequencing (BGI, Beijing, China). All primers used in this study are presented in Table S1. The minimum inhibitory concentrations (MICs) of CAZ-AVI (0.125/4-128/4 mg/L) and meropenem (0.125-128 mg/L) were determined using the broth microdilution method, in accordance with the guidelines of the Clinical Laboratory and Standards Institute (CLSI) [27]. *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as quality control strains for susceptibility testing [27].

Phenotypical identification of HMKP isolates

String tests were conducted to determine the hypermucoviscosity phenotype. The presence of the hypermucoviscosity phenotype was confirmed when a viscous string longer than 5 mm could be generated by stretching a single colony of the isolate on a Columbia blood agar plate (Dijing, Guangzhou, China) using an inoculation loop (positive). *K. pneumoniae* isolates that failed to generate longer than 5 mm of string in the test were classified as classic *K. pneumoniae* (negative) [28].

Mucoviscosity assay

Mucoviscosity was assessed by the mucoviscosity assay as described previously [29]. Briefly, overnight cultures were inoculated in fresh Luria Broth (LB) broth and grown to the logarithmic phase at 37 °C. The cultures were then resuspended in 1× phosphate-buffered saline (PBS) and adjusted to a concentration of 1 OD_{600}/mL. Subsequently, the suspensions were centrifuged at 1,000 *g* for 5 min, and the OD_{600} values of the supernatant were measured. The experiments were performed in triplicate. The supernatant of HMKP was expected to have a higher absorbance reading than classic *K. pneumoniae*, as HMKP produces more capsules, which prevents the formation of tight pellets.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was conducted to confirm the homology of A301 and A302 strains. The PFGE protocol followed the previously described methods [30]. Strains were classified as the same clone type if they exhibited ≥ 85% genetic similarity or displayed less than four fragment differences in the PFGE profiles.

In vitro selection of CAZ-AVI-resistant KPC-Kp

Strains included in this section were randomly selected based on their prevalence in China [31, 32]. The passage method employed in this study followed a previously published approach with minor modifications [15]. Briefly, a multipassage selection was conducted at low CAZ-AVI concentrations (1/2 × the MICs of parent isolates). Overnight cultures were inoculated at a ratio of 1/1000 in LB broth and incubated at 37 °C with shaking (220 rpm) for 16 h (Passage 1). Subsequently, the resulting cultures were inoculated at a ratio of 1/1000 into fresh LB (Passage 2), and this process was repeated daily for 60 passages. CAZ-AVI was included in all LB broths used during the passaging, maintained at a consistent concentration of half the MIC of the parent isolate. Changes in MICs during the passaging of the induced isolates were assessed every two passages using the broth microdilution method. After completion of 60 passages, all induced strains were stored at -80 °C. The nomenclature "parent strain name - induced resistance (IR)" (e.g., K1-IR) was used to label the CAZ-AVI-resistant isolates post-induction. To evaluate the stability of the phenotype, the four induced hypermucoid and CAZ-AVI-resistant KPC-Kp strains (K1-IR, K2-IR, K3-IR, K4-IR) were propagated for additional 20 passages without exposure to CAZ-AVI.

Whole-genome sequencing and genetic analysis

Whole-genome sequencing (WGS) was conducted for the parent isolates, their corresponding IR isolates, and clinical isolates A301 and A302, as previously described [15]. For each strain, single colonies from an overnight agar plate were cultured in 4 mL of LB broth at 37 °C for 16 h. Total DNA was then extracted using the FastPure Bacteria DNA Isolation Mini Kit (Vazyme, Nanjing, China). WGS was performed using a Novaseq 6000 platform (Illumina, San Diego, CA, USA). Raw reads obtained for each strain were trimmed and assembled into contigs using shovill [33]. The assembled contigs were annotated using Prokka [34]. Multi-locus sequence typing (MLST) was determined using MLST software [35]. Resistance genes and virulence genes were identified using ABRicate with the underlying database [36]. *wzi* typing methods were employed to determine the capsular serotypes (K) of the included isolates as previously described [26, 37]. To investigate single nucleotide polymorphisms (SNPs) between the parent isolates and their corresponding IR isolates, Snippy was utilized [38]. Furthermore, PCR and Sanger sequencing were performed to identify mutations in *wzc*.

Cloning of *wzc* variants and expression in wild-type strains

The cloning of both wild-type and mutated *wzc* into the vectors pBAD33 or pBAD33tet was conducted. The vector pBAD33tet was constructed by replacing the chloramphenicol resistance gene in pBAD33 with a tetracycline resistance gene from pACYC184 [39]. For each parent and IR-hypermucoviscous strain, the wild-type and mutated *wzc* genes were amplified by PCR using primers that containing 15-bp sequences at both ends, including the XbaI restriction site on PBAD33 or PBAD33tet (Table S1). The product was subsequently purified using a gel extraction kit (Takara, Dalian, China), and then cloned into the linearised plasmid pBAD33 or pBAD33tet, which had been digested with XbaI, using the In-Fusion® HD Cloning Kit (Takara, Dalian, China). The resulting constructs were confirmed by Sanger sequencing, and subsequently were transformed into the wild-type strains via electroporation. The expression of wild-type and mutant *wzc* was induced using 50 mM L-arabinose [39].

The real-time reverse transcriptase-polymerase chain reaction

To confirm the successful expression of the *wzc* gene carried by the vector in wild-type strains, the real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed. The experiments were performed in triplicate to ensure the reliability. Total bacterial RNA was extracted using Bacterial RNA Extraction Kit (Vazyme, Nanjing, China) following the manufacturer's instructions. The concentration and purity of the extracted total RNA were evaluated using a NANODROP 1000 spectrophotometer (Thermo Scientific, Massachusetts, America). To eliminate the genomic DNA contamination, RNase-free DNase I was utilized. Reverse

transcription was carried out using the HiScript III-RT SuperMix Kit (Vazyme, Nanjing, China). Subsequently, the qPCR was performed on a LightCycle® 96 (Roche, Basel, Switzerland) utilizing a ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and *wzc* primers (Table S1). The *rpoB* gene served as the reference (Table S1).

Virulence assay

The wax moth (*Galleria mellonella*) larvae assay used in this study was slightly adjusted based on a method previously described [40]. The larvae were obtained from Huiyude Biotechnology (Tianjin, China). PBS was used as a negative control. Single bacterial colonies were employed to inoculate in 5 mL of LB broth, which were then incubated at 37 °C with 200 rpm shaking until reaching the exponential phase. Subsequently, the cultures were adjusted to a turbidity equivalent to 10^8 colony-forming units (CFU)/mL in PBS, as determined by plate count. These cultures were further diluted 1/10 to obtain a final concentration of 10^7 CFU/mL. For each strain, three groups of larvae ($n=10$ in each group) were used. 20 µL of aliquots of the diluted cultures were injected into the last left proleg of the larvae using a Disposable Sterile Insulin Syringe U-100 (B.Braun, Germany). The infected larvae were subsequently maintained at 37 °C throughout the assay, and their survival was recorded at specific time points: 6, 12, 18, 24, 36, 48 and 72 h. Survival curves were generated and analyzed using GraphPad Prism 9 software.

Statistical analysis

Continuous data in this study are presented as means ± standard deviation (SD). Categorical variables were reported as frequencies and percentages. Statistical analysis was performed using the SPSS software package, version 26.0. The Student's *t*-test and Chi-square test were conducted to determine the significance of the observed differences. All tests were two-tailed, and *P* values < 0.05 were considered statistically significant.

Data summary

All WGS data have been deposited in the NCBI database. IR and the corresponding parent strains: BioProject accession [PRJNA1167439]. Isolates A301 and A302: BioSample accessions [SAMN33041006] and [SAMN33041032], under BioProject accession [PRJNA930962].

Results

The hypermucoviscous phenotypes in CAZ-AVI-sensitive and CAZ-AVI-resistant clinical KPC-Kp isolates

A total of 15 CAZ-AVI-resistant and 248 CAZ-AVI-sensitive clinical KPC-Kp strains were included in this study. Among the 15 CAZ-AVI-resistant strains, 6 belonged to the K47 serotype and 9 belonged to the K64 serotype. Among the 248 CAZ-AVI-sensitive strains, 41 belonged to K47, 171 belonged to K64, 14 belonged to K25, and 22 belonged to other serotypes (as shown in Table 1).

The string test was performed to evaluate the hypermucoviscous phenotype. Among the CAZ-AVI-resistant KPC-Kp isolates, 46.7% (7/15) tested positive in the string test, indicating the presence of hypermucoviscosity. In contrast, among the CAZ-AVI-sensitive KPC-Kp isolates, only 5.6% (14/248) showed a positive string test and were identified as hypermucoviscosity. The proportion of isolates demonstrating the hypermucoviscous phenotype was significantly lower among the CAZ-AVI-sensitive strains compared to CAZ-AVI-resistant strains ($P<0.001$, as indicated in Table 1). The mucoviscosity assay confirmed that the mucoviscosity of isolates with a positive string test was significantly higher than that of isolates with a negative string test, whether the isolates resistant to CAZ-AVI or not. (Table 1).

Notably, of all the collected KPC-Kp isolates, two isolates, A301 (CAZ-AVI-sensitive with a negative string test) and A302 (CAZ-AVI-resistant with a positive

Table 1 Information of CAZ-AVI-resistant and -sensitive KPC-Kp isolates

	CAZ-AVI-resistant isolates ($n=15$)		**CAZ-AVI-sensitive isolates ($n=248$)**	
	Isolates with negative string test (n, %)	Isolates with positive string test (n, %)	Isolates with negative string test (n, %)	Isolates with positive string test (n, %)
Serotypes				
K64	4(26.7%)	5(33.3%)	159(64.1%)	12(4.8%)
K47	4(26.7%)	2(13.3%)	39(15.7%)	2(0.8%)
K25	0(0.0%)	0(0.0%)	14(5.6%)	0(0.0%)
other	0(0.0%)	0(0.0%)	22(8.9%)	0(0.0%)
total	8(53.3%)	7(46.7%)	234(94.4%)	14(5.6%)
Mucoviscosity assays (means ± SD)	0.102 ± 0.011	0.457 ± 0.099	0.136 ± 0.049	0.434 ± 0.141

Note: Continuous data are presented as means ± standard deviation (SD). Categorical variables were reported as frequencies (n) and percentages (%)

string test), were obtained from the same patient who unfortunately had a fatal outcome. The mucoviscosity assay revealed a higher mucus production in A302 (0.525 ± 0.002) compared to A301 (0.141 ± 0.001, $P < 0.001$). The homology between A301 and A302 was validated using PFGE, as shown in Figure S1. More characteristics of A301 and A302 are presented in Table S2. Both A301 and A302 isolates belonged to ST11-K47 clone and were isolated from sputum and urine samples, respectively. To explore the molecular mechanism of the transition from CAZ-AVI-sensitive KPC-Kp isolates into CAZ-AVI-resistant isolates exhibiting a hypermucoviscous phenotype, a more thorough analysis of strains A301 and A302, along with subsequent in vitro induction strains, will be conducted.

The transition of hypermucoviscous phenotypes in KPC-Kp during in vitro selection of CAZ-AVI-resistance and the stability of induced hypermucoviscous phenotypes

To further investigate the association between hypermucoviscous phenotypes and CAZ-AVI-resistance, ten CAZ-AVI-resistant IR KPC-Kp isolates were generated through passaging in vitro. The parent KPC-Kp isolates included in this study exhibited negative string test results. The specimen types and molecular characteristics of these isolates are shown in Table S3. During the string test, it was observed that four out of the ten IR strains (K1-IR, K2-IR, K3-IR, and K4-IR) transitioned from negative to positive, indicating the acquisition of a hypermucoviscous phenotype. Mucoviscosity assays revealed that all four induced hypermucoviscous IR isolates exhibited a more than two-fold increase in mucoviscosity compared to the parent strains (all $P < 0.01$, Table 2). Among the four hypermucoviscous IR isolates, K2 and K3 belonged to ST11-K64, K1 belonged to ST11-K47, and K4 belonged to ST15-K19 (Table S3).

To assess the stability of the hypermucoviscous phenotype, the four induced hypermucoviscous and CAZ-AVI-resistant KPC-Kp strains were subjected to 20 passages without CAZ-AVI. The results demonstrated that all four strains maintained positive string test results, indicating the persistent expression of the hypermucoviscous phenotype (Table 2). Additionally, there were no significant differences in mucoviscosity between the four hypermucoviscous isolates and the strains from the 60th generation.

Mutations in Wzc protein were found in CAZ-AVI-resistant hypermucoviscous KPC-Kp in vitro and in vivo

To investigate the mechanism underlying CAZ-AVI resistance and bacterial hypermucoviscosity, mutations that occurred during the in vitro selection and between A301 and A302 were identified using WGS. The resistance and virulence gene profiles of ten parent KPC-Kp strains were showed in Figure S2 and S3. The identified mutations are listed in Table 3 and Table S4. Notably, regarding the genes associated with bacterial hypermucoviscosity, various mutations were observed in the Wzc protein of all four IR-hypermucoviscous strains (K1-IR, P644L; K2-IR, D567E; K3-IR, P647L; K4-IR, T569P) in comparison to the parent strains. Conversely, the remaining six IR strains with a negative string test did not exhibit any mutations in the Wzc protein. In addition, strain A302 exhibited a mutation in Wzc protein (S715*)

Table 2 Phenotypes of KPC-Kp isolates at baseline, after 60 passages with CAZ-AVI exposure, and additional 20 passages without CAZ-AVI exposure

Strains	CAZ-AVI MIC (mg/L)[a]			String test[a,b]			Mucoviscosity assay[a]		
	Passage 0	Passage 60	Passage 80	Passage 0	Passage 60	Passage 80	Passage 0 (means ± SD)	Passage 60 (means ± SD)	Fold change (P60/P0)[c] (means ± SD)
K1	4	32	32	-	+	+	0.090 ± 0.001	0.238 ± 0.001	2.641 ± 0.047
K2	4	32	32	-	+	+	0.158 ± 0.002	0.354 ± 0.001	2.234 ± 0.029
K3	4	32	32	-	+	+	0.103 ± 0.001	0.609 ± 0.008	5.911 ± 0.152
K4	0.5	16	16	-	+	+	0.097 ± 0.001	0.462 ± 0.002	4.767 ± 0.030
K5	4	128	128	-	-	/	0.115 ± 0.015	0.125 ± 0.007	1.106 ± 0.153
K6	2	16	16	-	-	/	0.094 ± 0.001	0.093 ± 0.002	0.991 ± 0.024
K7	4	32	32	-	-	/	0.101 ± 0.004	0.095 ± 0.002	0.974 ± 0.045
K8	4	16	16	-	-	/	0.096 ± 0.001	0.096 ± 0.002	1.002 ± 0.025
K9	2	16	16	-	-	/	0.094 ± 0.001	0.093 ± 0.002	0.990 ± 0.033
K10	1	128	128	-	-	/	0.099 ± 0.003	0.093 ± 0.012	1.106 ± 0.151

[a] Passage 0: wild-type (parent) KPC-Kp strains before in vitro passages; Passage 60: induced KPC-Kp strains after 60 passages with CAZ-AVI exposure; Passage 80: induced KPC-Kp strains after 60 passages with CAZ-AVI exposure and additional 20 passages without CAZ-AVI exposure

[b] "-" means negative for the string test; "+" means positive for the string test; "/" means that only the 4 strains (K1, K2, K3, and K4) that changed from negative to positive for the string tests after 60 passages with CAZ-AVI exposure were further passaged for an additional 20 generations without CAZ-AVI exposure, after which string tests were performed

[c] The fold change in the mucoviscosity assay was obtained by dividing the mucoviscosity assay results of the strains after 60 passages with CAZ-AVI exposure by the mucoviscosity assay results of the wild type strains

Table 3 Mutations of IR-hypermucoviscous strains in vitro selection compared to parent strains

Strains	String test of IR strains[a]	Proteins	Mutations	Protein annotation	Change of MICs (passage 0→passage 60, mg/L)
K1	+	Ssb	A78V	Plasmid-derived single-stranded DNA-binding	4→32
		RffH	I5V	Glucose-1-phosphate thymidylyltransferase	
		Wzc	P644L	Capsule polysaccharide synthesis	
K2	+	HP	A20E	Hypothetical protein	4→32
		Wzc	D567E	Capsule polysaccharide synthesis	
K3	+	**Wzc**	P647L	Capsule polysaccharide synthesis	4→32
		MalT	Q237-A240del	HTH-type transcriptional regulator	
		HP	A20E	Hypothetical protein	
K4	+	MalT	E359K	HTH-type transcriptional regulator	0.5→16
		LamB	R374S	Maltoporin	
		Wzc	T569P	Capsule polysaccharide synthesis	

[a] "+" means positive for the string test

compared to strain A301 (Table S2). In terms of the genes associated with CAZ-AVI resistance, no KPC mutation was detected in the four IR-hypermucoviscous strains. In contrast, four out of the other six strains did display KPC mutations (K5, L169P+S181 ins; K6, E165_L166ins; K8, A172V; K9, E166_L167del).

To examine the correlation between CAZ-AVI resistance, the hypermucoviscous phenotype, and Wzc mutation in IR-hypermucoviscous strains during induction, the dynamic changes in CAZ-AVI MIC values, string test, and mutations in Wzc were summarized (Fig. 1). Isolates K1, K2, K3 and K4 transitioned from CAZ-AVI-sensitive to CAZ-AVI-resistant at passages 4, 22, 10 and 50, respectively. Subsequent to the emergence of resistance, the string tests of these strains changed from negative to positive, and each accompanied by mutations of Wzc protein.

Wzc mutations were also determined through PCR and Sanger sequencing in 15 CAZ-AVI-resistant and 248 CAZ-AVI-sensitive clinical KPC-Kp isolates included in this study. The results demonstrated that Wzc mutations occurred at various loci (A541T, P647S, S715*, C716Y and P648T) in five out of seven CAZ-AVI-resistant hypermucoviscous KPC-Kp (including strain A302). Totally, KPC mutations were identified in four out of the 15 CAZ-AVI-resistant strains [D179Y (n=3); A172V (n=1)]. Notably, no mutations in KPC was identified in any of the five hypermucoviscous isolates, consistent with the findings obtained from the IR-hypermucoviscous strains. Conversely, among the 14 CAZ-AVI-sensitive hypermucoviscous KPC-Kp strains, 6 harboured mutations in Wzc protein at different loci (S531L, A647V, G526A, Y572H, and G536A).

The role of mutations in *wzc* gene in hypermucoviscous phenotype and virulence

The wild-type and mutated *wzc* were cloned into the four parent strains and strain A301 to investigate the role of *wzc* gene mutations in the hypermucoviscous phenotype and virulence. RT-qPCR was performed to validate the successful expression of the wild-type and mutated *wzc* genes in the corresponding wild-type strains. The results demonstrated a significant increase in the expression levels of both wild-type and mutant *wzc* genes in the complemented strains compared to the wild-type strains with or without the empty vector (Fig. 2), confirming the successful construction of the complemented strains.

All wild-type KPC-Kp isolates exhibited enhanced mucus production when transformed with mutant *wzc* gene compared with the wild-type *wzc* gene (Table 4; Fig. 2). The string test results showed that all five wild-type isolates tested negative when carring the wild-type *wzc* gene and positive when carring the mutant *wzc* gene, indicating the acquisition of a hypermucoviscous phenotype due to the presence of the mutated *wzc* gene. Mucoviscosity assay further revealed that five wild-type isolates (K1,K2, K3, K4 and A301) carrying the mutant *wzc* gene displayed a 4-fold, 3-fold, greater than 3-fold, 7-fold and 2-fold increase in mucus production, respectively, compared to strains with the wild-type *wzc* gene (Table 4; Fig. 2). Conversely, there was no significant difference in the MIC values of CAZ-AVI between the strains transformed with the mutant and wild-type *wzc* genes (Table 5).

In a wax moth larvae infection model, the survival rate of larvae infected with the four parent strains and strain A301 expressing the mutant *wzc* gene was significantly lower compared to those infected with the strains expressing the wild-type *wzc* gene (Fig. 2) (P<0.001). After 24 h of infection, the mortality rates of wax moth larvae infected with strains K1, K2, K3, K4 and A301

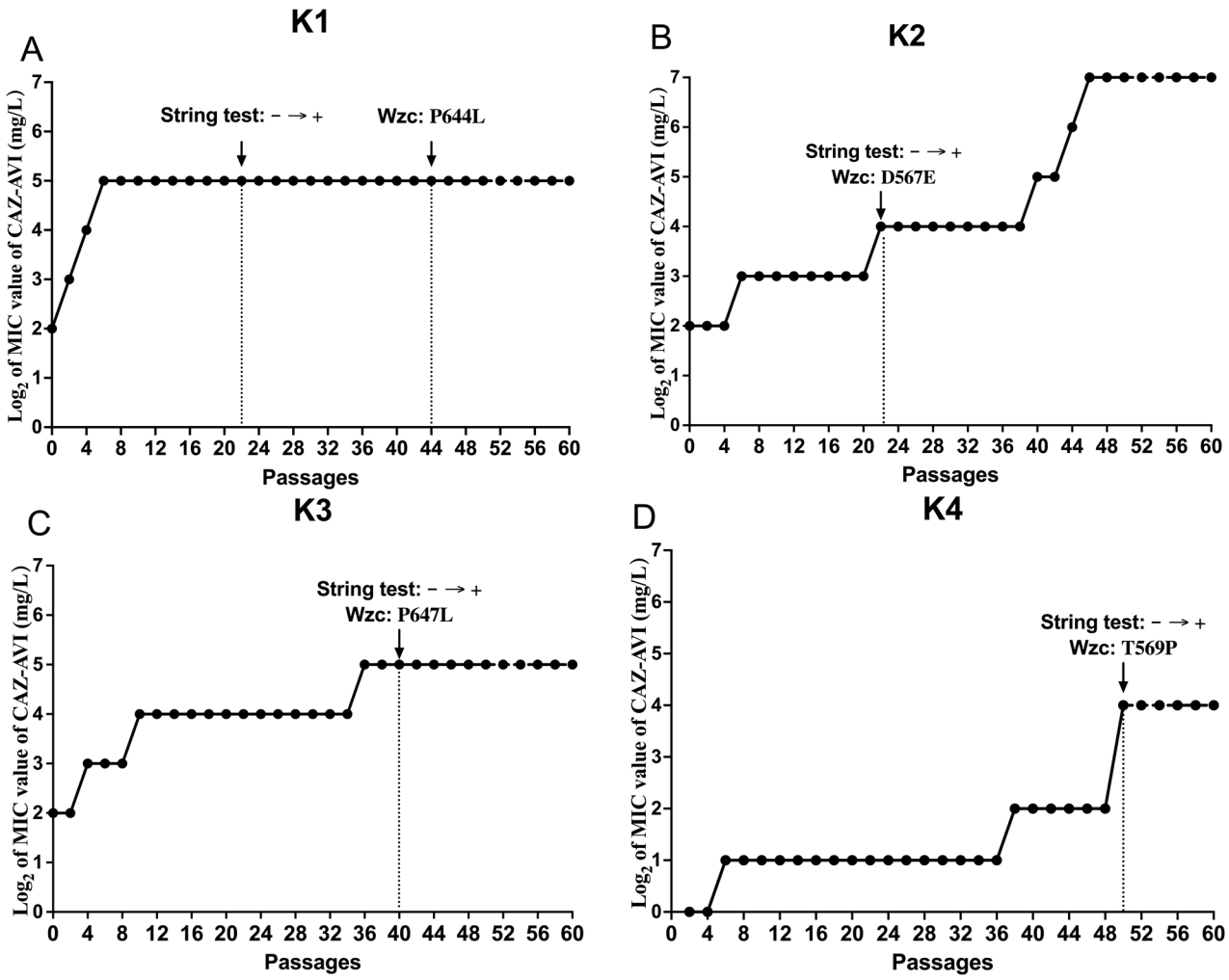

Fig. 1 Log_2 of MIC values of CAZ-AVI, string test and mutations in Wzc during passaging. The horizontal axis is the number of induction passages. The vertical axis is the Log_2 of MIC values of CAZ-AVI (mg/L). String test: - → + : during this generation, we observed a transition in the string test results from negative (-) to positive (+), indicating a significant change

carrying the mutant *wzc* were 90.0%, 80.0%, 60.0%, 90.0% and 50.0%, respectively. In contrast, the mortality rates after 24 h of infection with strains K1, K2, K3, K4 and A301 carrying the wild-type *wzc* gene significantly reduced ($P<0.01$), to 20.0%, 40.0%, 40.0%, 60.0% and 10.0%, respectively.

Discussion

In this study, we have, for the first time, revealed the underlying association between CAZ-AVI resistance and hypermucoviscosity in KPC-Kp isolates through the examination of clinical isolates and the evolution of laboratory strains. Our findings indicate a higher prevalence of hypermucoviscosity among KPC-Kp strains in the CAZ-AVI-resistant group compared to the CAZ-AVI-sensitive group. Furthermore, four out of ten CAZ-AVI-sensitive strains developed resistance to CAZ-AVI and acquired the hypermucoviscosity phenotype during passaging, indicating that the acquisition of CAZ-AVI resistance in KPC-Kp may often be accompanied by bacterial hypermucoviscosity. Additionally, various clone types of KPC-Kp strains, such as ST11-K47, ST11-K64, and ST15-K19, exhibited a hypermucoviscous phenotype during the development of CAZ-AVI resistance. These findings suggest that the coexistence of CAZ-AVI resistance and hypermucoviscosity may be widespread in KPC-Kp strains.

It was discovered that all IR strains exhibiting induced CAZ-AVI resistance and hypermucoviscous phenotype, as well as a majority of clinically isolated CAZ-AVI-resistant hypermucoviscous KPC-Kp strains, had mutations in the *wzc* gene. This was revealed through analysis of the mechanism underlying the hypermucoviscous phenotype. Functional verification of Wzc mutations revealed a significant increase in mucus production in the strains when transformed with mutant *wzc* genes.

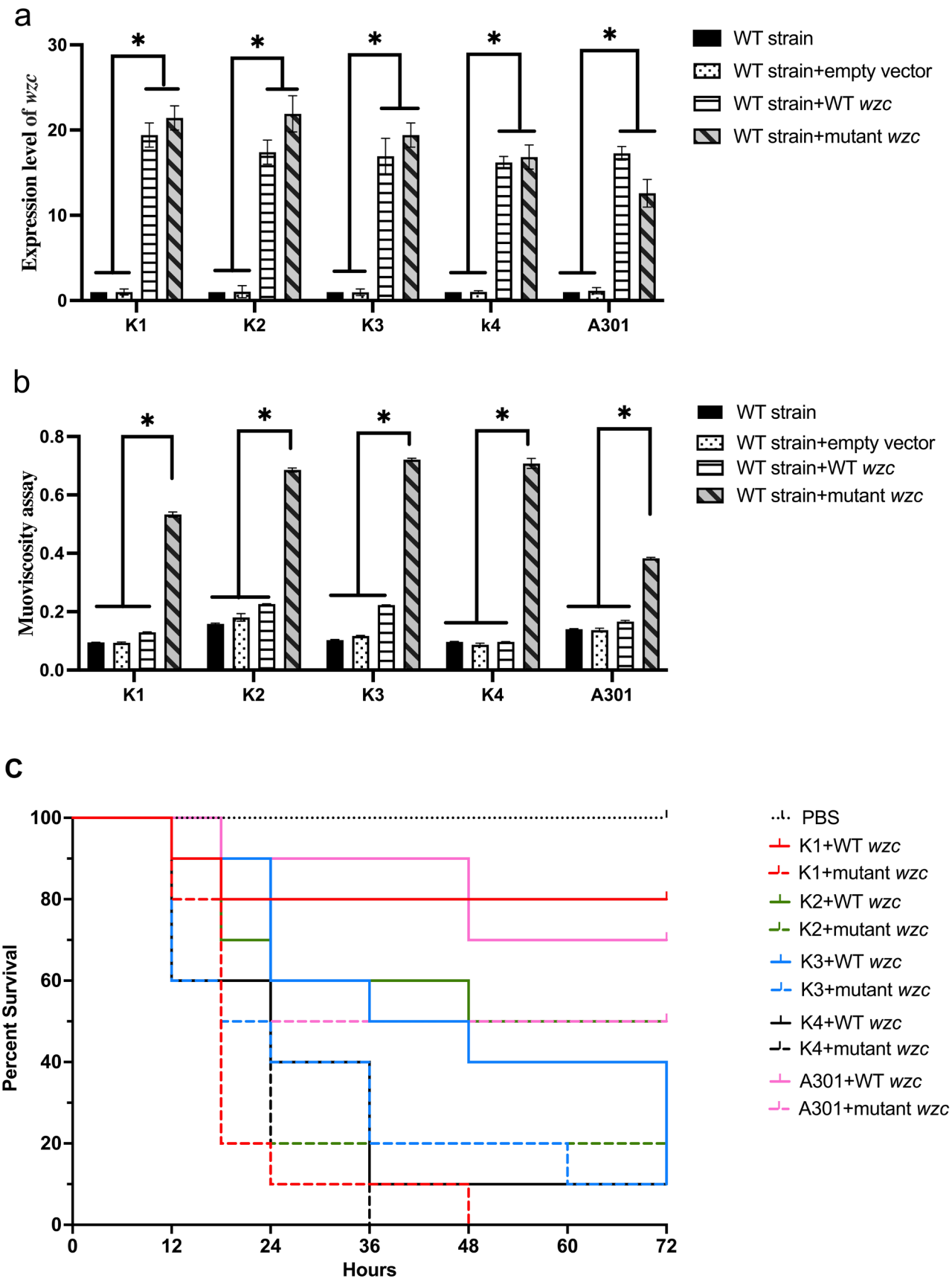

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 SNPs in *wzc* confer hypermucoviscosity and increased virulence. (**a**) *wzc* gene expression level changes in strains involved in functional validation of *wzc*. (**b**) SNPs in wzc confer hypermucoviscosity. The horizontal axis contains the names of strains. The vertical axis indicates the results of expression level of *wzc* and mucoviscosity assay (OD_{600} values) in triplicate. *: $P < 0.05$ (Student's *t*-tests). WT, wild-type. (**c**) Survival curves for wax moth (*Galleria mellonella*) larvae infected with ~ 10^7 CFU/mL (20 µL) of different isolates with wild-type or mutant *wzc* genes. PBS was used as a negative control. The curves represent the mean of 3 repeats (10 larvae in each repeat)

This confirmed that the hypermucoviscous phenotype observed in CAZ-AVI-resistant isolates was associated with mutations in the *wzc* gene. Analysis of homologous strains A301 and A302, isolated from the same patient, indicated that developed CAZ-AVI resistance in KPC-Kp strains may occur in vivo along with mucus overproduction, posing a serious clinical risk.

While genes such as *wzi* and *wzy*, which are part of the same operon as *wzc*, might also impact CPS production, our study primarily focus on investigating the effects of Wzc mutation due to its clear association with hypermucoviscous phenotype here. The Wzc protein is considered as the major regulator of CPS production in gram-negative bacteria, but the specific mechanism driving this process remains unclear [41]. Further research is needed to elucidate the intrinsic mechanism by which Wzc mutations lead to bacterial hypermucoviscosity. Mutations in Wzc are infrequent and have seldom been reported. Previous studies have indicated that mutations in Wzc resulted in increased hypermucoviscous properties in *K. pneumoniae* and *Acinetobacter baumannii*, which are consistent with our observations [39, 42]. We also examined the mutation sites of Wzc protein, revealing that all the mutations occurred within the C-terminal domain (from R446 to K720) in our study, which is essential for capsule assembly [43, 44]. This further affirmed the significance of the C-terminal domain in CPS synthesis.

Furthermore, our study confirmed that Wzc mutations lead to increased virulence of KPC-Kp strains. Previous studies have confirmed that bacterial hypermucoviscosity resulting from Wzc mutations in *K. pneumoniae* led to significant phagocytosis resistance in macrophages and enhanced lethality in murine models of urinary tract infection [39]. The hypermucoviscous strain A302, isolated from the urine specimen, may possess enhanced lethality, potentially contributing to the rapid death of the patient. The increased virulence caused by the Wzc mutation further exacerbates the public health burden associated with strains carrying mutant Wzc.

Our study does not provide evidence that Wzc mutations or hypermucoviscosity directly contributes to CAZ-AVI resistance. This suggests that the evolution of CAZ-AVI resistance and the development of hypermucoviscosity phenotype may occur through independent mechanisms. Additionally, our results indicate a predisposition that hypermucoviscosity and Wzc mutations occurred in CAZ-AVI-resistant strains without KPC mutations (a significant mechanism of the CAZ-AVI resistance in KPC-Kp) [7]. These findings apply to both IR-hypermucoviscous strains induced in vitro and clinically isolated CAZ-AVI-resistant hypermucoviscous strains. Therefore, additional studies are required to further investigate other factors involved in the mechanisms of CAZ-AVI resistance and hypermucoviscosity.

Conclusions

In summary, our study confirms that resistance to CAZ-AVI in KPC-Kp may be accompanied by bacterial hypermucoviscosity in both clinical and laboratory strains, with mutations in the CPS synthesis gene *wzc* often playing an important role in this process. Clinicians should remain vigilant regarding the emergence of CAZ-AVI-resistant hypermucoviscous KPC-Kp strains during medication, as this poses a potential threat to public health.

Table 4 Role of mutations in *wzc* gene

Transformed plasmid	Strains	String test[a]				Mucoviscosity assay				
		WT strain	WT strain + empty vector	WT strain + WT *wzc*	WT strain + mutant *wzc*	WT strain	WT strain + empty vector	WT strain + WT *wzc* (means ± SD)	WT strain + mutant *wzc* (means ± SD)	Fold change (WT strain + mutant *wzc*/WT strain + WT *wzc*, means ± SD)
pBAD33tetP644L	K1	-	-	-	+	0.090 ± 0.001	0.093 ± 0.003	0.130 ± 0.001	0.533 ± 0.007	4.097 ± 0.053
pBAD33D567E	K2	-	-	-	+	0.158 ± 0.002	0.180 ± 0.012	0.226 ± 0.001	0.685 ± 0.006	3.028 ± 0.042
pBAD33P647L	K3	-	-	-	+	0.103 ± 0.001	0.117 ± 0.002	0.223 ± 0.001	0.721 ± 0.003	3.240 ± 0.009
pBAD33tetT569P	K4	-	-	-	+	0.097 ± 0.001	0.087 ± 0.005	0.097 ± 0.001	0.708 ± 0.014	7.326 ± 0.201
pBAD33S715*	A301	-	-	-	+	0.141 ± 0.001	0.137 ± 0.002	0.160 ± 0.002	0.383 ± 0.002	2.396 ± 0.040

Note: Target fragments of WT and mutant *wzc* contained in the recombinant plasmids were obtained from each WT and IR-hypermucoviscous strain, respectively. WT, wild type

[a] "-" means negative for the string test; "+" means positive for the string test

Table 5 MIC values of CAZ-AVI between strains transformed with the mutant and wild-type *wzc* genes

Strains	MIC values of CAZ-AVI (mg/L)			
	WT strain	WT strain + empty vector	WT strain + WT *wzc*	WT strain + mutant *wzc*
K1	4	4	4	4
K2	4	4	4	4
K3	4	4	4	4
K4	0.5	1	1	1
A301	1	1	1	1

Note: Target fragments of WT and mutant *wzc* contained in the recombinant plasmids were obtained from WT and IR-hypermucoviscous strains, respectively. WT, wild-type

Abbreviations

K. pneumoniae	Klebsiella *pneumoniae*
CRKP	Carbapenem-resistant Klebsiella *pneumoniae*
KPC	Klebsiella *pneumoniae* carbapenemase
NDM	New Deli metallo-beta-lactamase
IMP	Imipenemase
VIM	Verona integron-encoded metallo-beta-lactamase
OXA	Oxacillinase
AVI	Avibactam
CAZ-AVI	Ceftazidime-avibactam
KPC-Kp	KPC-producing *K. pneumoniae*
HMKP	Hypermucoviscous *K. pneumoniae*
CPS	Capsule polysaccharide
rmpA	Regulator of mucoid phenotype A
CR-HMKP	Carbapenem-resistant hypermucoviscous *K. pneumoniae*
PCR	Polymerase chain reaction
MICs	Minimum inhibitory concentrations
CLSI	Clinical Laboratory and Standards Institute
LB	Luria Broth
PFGE	Pulsed-field gel electrophoresis
IR	Induced resistance
WGS	Whole-genome sequencing
MLST	Multi-locus sequence typing
SNPs	Single nucleotide polymorphisms
RT-qPCR	Real-time reverse transcriptase-polymerase chain reaction
PBS	Phosphate-buffered saline
CFU	Colony-forming units
SD	Standard deviation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03508-w.

Supplementary Material 1: Table S1. List of primers used in the study. **Table S2.** Characteristics of strains A301 and A302. MLST: multi-locus sequence typing; KPC: Klebsiella *pneumoniae* carbapenemase; CAZ-AVI: Ceftazidime-avibactam; CAZ: Ceftazidime; MEM: Meropenem; AMP: Ampicillin; ATM: Aztreonam; PB: Polymyxin B; GM: Gentamicin; LVX: Levofloxacin; CRO: Ceftriaxone; TGC: Tigecycline. The MIC for tigecycline was determined in accordance with the standards of the US Food and Drug Administration. The differences between A301 and A302 are shown in bold. **Table S3.** Characteristics of classical KPC-Kp strains. KPC, Klebsiella *pneumoniae* carbapenemase; MLST: multi-locus sequence typing; CAZ-AVI: Ceftazidime-avibactam. [a]WT: wild-type; Premature stop codon: a premature stop codon at amino acid position 63 in OmpK35; 134 to 135 GD insertion: 134 to 135 GD insertion in OmpK36. **Table S4.** Mutations of IR (non-hypermucoviscous) strains in vitro selection compared to parent strains.

Supplementary Material 2: Figure S1. PFGE of strain A302/A301 and other 10 randomly selected clinical KPC-Kp isolates.

Supplementary Material 3: Figure S2. Heat map of resistance genes carried by 10 wild-type KPC-Kp strains.

Supplementary Material 4: Figure S3. Heat map of virulence genes carried by 10 wild-type KPC-Kp strains.

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Author contributions
Conceptualization, YYG, ZWL and CZ; Methodology, JW and LKY; Investigation, CYZ, XY, FFL and JHL; Writing—Original Draft, YYG and JW; Writing—Review and Editing, YYG, JW, YJW, YZ and ZWL; Supervision, BML, NHH, JKC and SNX; Funding Acquisition, CZ. All authors read and approved the final manuscript.

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Data availability
All WGS reads are available from the NCBI database. IR and the corresponding parent strains: BioProject accession [PRJNA1167439] (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1167439). Isolates A301 and A302: BioSample accession [SAMN33041006] (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN33041006) and [SAMN33041032] (https://www.ncbi.nlm.nih.gov/biosample/?term=SAMN33041032). Other data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate
Our study was approved by The First Affiliated Hospital of Guangzhou Medical University Ethics Committee (approval number: ES-2023-057-01). According to The First Affiliated Hospital of Guangzhou Medical University Ethics Committee, the need for informed consent was deemed unnecessary because data were collected from routine analysis.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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