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**RPD3 and UME6 are involved in the activation of PDR5 transcription and pleiotropic drug resistance in ρ⁰ cells of *Saccharomyces cerevisiae***

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**Abstract**

**Background:** In *Saccharomyces cerevisiae*, the retrograde signalling pathway is activated in ρ⁰/− cells, which lack mitochondrial DNA. Within this pathway, the activation of the transcription factor Pdr3 induces transcription of the ATP-binding cassette (ABC) transporter gene, *PDR5*, and causes pleiotropic drug resistance (PDR). Although a histone deacetylase, Rpd3, is also required for cycloheximide resistance in ρ⁰/− cells, it is currently unknown whether Rpd3 and its DNA binding partners, Ume6 and Ash1, are involved in the activation of *PDR5* transcription and PDR in ρ⁰/− cells. This study investigated the roles of *RPD3*, *UME6*, and *ASH1* in the activation of *PDR5* transcription and PDR by retrograde signalling in ρ⁰ cells.

**Results:** ρ⁰ cells in the *rpd3*∆ and *ume6*∆ strains, with the exception of the *ash1*∆ strain, were sensitive to fluconazole and cycloheximide. The *PDR5* mRNA levels in ρ⁰ cells of the *rpd3*∆ and *ume6*∆ strains were significantly reduced compared to the wild-type and *ash1*∆ strain. Transcriptional expression of *PDR5* was reduced in cycloheximide-exposed and unexposed ρ⁰ cells of the *ume6*∆ strain; the transcriptional positive response of *PDR5* to cycloheximide exposure was also impaired in this strain.

**Conclusions:** *RPD3* and *UME6* are responsible for enhanced *PDR5* mRNA levels and PDR by retrograde signalling in ρ⁰ cells of *S. cerevisiae*.

**Keywords:** *Saccharomyces cerevisiae*, *UME6*, *RPD3*, Pleiotropic drug resistance, ρ⁰ cells, Retrograde signalling

**Background**

In the yeast, *Saccharomyces cerevisiae*, multidrug resistance can result from the overexpression of plasma membrane-localized ABC transporters, such as Pdr5, Snq2, and Yor1. Pdr5 is a major efflux pump of functionally and structurally unrelated antifungal drugs or compounds, such as fluconazole and cycloheximide [1]. Expression of *PDR5* can be induced by the paralogous Zn2Cys6 transcription factors, Pdr1 and Pdr3. Pdr1 and Pdr3 can form both homo- and heterodimers, and directly bind structurally diverse drugs and xenobiotics [2, 3]. *PDR5* has four perfect and degenerate pleiotropic drug response elements (PDREs) in its promoter region [4]. Pdr1 and Pdr3 are constitutively bound to PDRE in the *PDR5* promoter [3]. Although *PDR1* does not have a PDRE in its promoter region, *PDR3* has two PDREs, and is thereby regulated by an autoregulatory loop involving Pdr3 [5]. Thus, *PDR3* is transcriptionally regulated by both Pdr1 and Pdr3 via PDRE [5]. Gain-of-function mutations in Pdr1 and Pdr3 lead to hyperactive
transcription of ABC transporter genes, such as PDR5, SNQ2, and YOR1, resulting in the induction of pleiotropic drug resistance (PDR) [6, 7].

Although PDR1 and PDR3 have functionally redundant roles in PDR, PDR1 plays a predominant role in PDR to toxic agents and basal PDR5 expression [8, 9]. Deletion of PDR1 increases susceptibility to cycloheximide compared to PDR3, whereas the disruption of PDR1 and PDR3 causes hypersensitivity to cycloheximide and oligomycin compared with a single disruption [9].

PDR3 plays a predominant role in the PDR of ρ0− cells without mitochondrial DNA in S. cerevisiae [10]. The retrograde signalling pathway is strongly activated in ρ0− cells of S. cerevisiae and Candida glabrata, and the expression of multidrug resistance genes, including PDR5 and CgCdr1 is induced. The induction of PDR5 in this retrograde signalling pathway requires Pdr3 but not Pdr1 [10]. In the retrograde signalling pathway, Pdr3 directly interacts with the Hsp70 chaperone Ssa1, and Pdr3 activity is inhibited [11]. Intriguingly, this Pdr3-Ssa1 association is decreased in ρ0− cells, and Pdr3 activity is strongly stimulated [11].

In S. cerevisiae, small (0.6 MDa) (Rpd3S) and large (1.2 MDa) (Rpd3L) corepressor complexes exist, share specific subunits such as a histone deacetylase, Rpd3, Ume1, and Sin3 [12]. They participate in chromatin remodelling and transcriptional repression [13, 14]. Dep1 and Sds3 are specific subunits of the Rpd3L complex, whereas the Rpd3S complex contains two unique subunits, Rco1p and Eaf3p [12]. Rpd3 is a histone deacetylase, while Sds3 is involved in transcriptional silencing and sporulation. The Rpd3L complex contains the DNA binding transcription factors, Ume6 and Ash1, which are responsible for the targeted deacetylation of gene promoters [12]. For example, Ume6 binds the URS1 upstream regulatory sequence on the INO1 promoter and represses INO1 expression by recruiting Rpd3 via the corepressor Sin3 and chromatin remodelling factor Isw2 [14, 15]. Although both Ume6 and Ash1 are bound to the INO1 and HO promoters, Ume6 specifically represses INO1 gene expression, and Ash1 specifically inhibits HO gene expression [12]. UME6 is known to repress carbon/nitrogen metabolic and early meiotic gene expression while participating in gene activation [16].

Borecka-Melkusova et al. have shown that Rpd3 is required for basal PDR5 transcription and Pdr3-mediated PDR [17]. In addition, the sds3Δ, dep1Δ, and rpd3Δ strains are sensitive to drugs, indicating that the Rpd3L complex is involved in PDR [17]. In contrast to sds3Δ, dep1Δ, and rpd3Δ strains, the uma6Δ and ash1Δ strains displayed no sensitivity to cycloheximide at the minimum inhibitory concentration [17]. In addition, Yilmantasiri et al. have also reported that the una6Δ strain does not confer sensitivity to a range of fungicides including cycloheximide, ketoconazole, fluconazole, oligomycin, and benomyl when tested in a spot dilution assay [18]. The authors also reported that deletion of UME6 does not reduce Pdr5 expression in western blot analysis [18]. Robbins et al. reported that the decreased azole resistance in the rpd3Δ strain of S. cerevisiae does not result from downregulation of PDR5 mRNA [19]. Rather, it results from diminished Hsp90-dependent antifungal drug resistance in Candida albicans and S. cerevisiae [19]. Furthermore, Jensen et al. reported that artemisinin sensitivity in the rpd3Δ strain of S. cerevisiae occurred due to the impaired endoplasmic reticulum (ER) to Golgi trafficking of Pdr5, and not from transcriptional down-regulation of PDR5 [20].

As mentioned above, PDR5 expression or PDR in the rpd3Δ, uma6Δ, and ash1Δ mutant strains, has been examined mainly in ρ+ cells with mitochondrial DNA, but not in ρ0− cells. However, Borecka-Melkusova et al. showed that ρ0− cells in the rpd3Δ strain also have significantly lower cycloheximide resistance than those in the wild-type [17]. Although PDR5 transcription and PDR are activated by retrograde signalling via Pdr3 in ρ0− cells, whether RPD3, UME6, and ASH1 are involved in the activation of PDR5 transcription and PDR in ρ0− cells has not yet been examined. Therefore, this study investigated the roles of RPD3, UME6, and ASH1 in the activation of PDR5 transcription and PDR by retrograde signalling in ρ0− cells.

**Results**

**Susceptibility of ρ0− cells in strain uma6Δ to fluconazole and cycloheximide**

To investigate whether UME6 and ASH1 are involved in the PDR of ρ0− cells, the sensitivity of ρ0− cells in the uma6Δ and ash1Δ mutant strains to fluconazole and cycloheximide was examined using a spot dilution assay. ρ0− cells in the uma6Δ::bleMX6, pdr3Δ::bleMX6, and rpd3Δ::bleMX6 mutant strains were more sensitive to fluconazole and cycloheximide than those in the wild-type, ash1Δ::bleMX6, and gat3Δ::bleMX6 strains (Fig. 1). However, ρ0− cells of the uma6Δ mutant strain were less susceptible to fluconazole and cycloheximide than those in the pdr3Δ and rpd3Δ mutant strains (Fig. 1). Similar results were also observed for ρ0− cells in the wild-type, uma6Δ::KanMX, pdr3Δ::KanMX, and rpd3Δ::KanMX, ash1Δ::KanMX, and gat3Δ::KanMX strains (data not shown). We also obtained similar results in ρ0− cells of the uma6Δ mutant derived from the W303−1A strain (data not shown). Since fluconazole and cycloheximide are functionally and structurally unrelated compounds, these results suggest that UME6, but not ASH1, is responsible for the activation of PDR5 transcription and PDR in ρ0− cells.
cells of *S. cerevisiae*. However, it cannot be ruled out that *ASH1* may be responsible for resistance to other drugs in ρ0 cells of *S. cerevisiae*.

In the spot dilution assay, the ρ0 cells in the *ume6Δ* mutant displayed less susceptibility to fluconazole and cycloheximide than in the *pdr3Δ* and *rpdr3Δ* mutants. Therefore, we further investigated whether *UME6* is responsible for PDR in ρ0 cells using a co-cultivation assay [21]. As ρ0 cells of the *ume6Δ* mutant, but not of the *ash1Δ* and *gat3Δ* mutants, were more sensitive to fluconazole and cycloheximide than those in the wild-type strain, *ash1Δ* and *gat3Δ* mutants were used as controls for the *ume6Δ* mutant in the co-cultivation assay (Fig. 1). ρ0 cells in two mutant strains, *gat3Δ::KanMX* and *ume6Δ::bleMX6*, were co-cultivated in the presence and absence of 100 μg/mL fluconazole. The number of viable cells of each mutant strain in the co-culture was estimated by spreading them onto yeast extract peptone dextrose (YPD) plates containing G418 or Zeocin. We found that ρ0 cells in the *ume6Δ::bleMX6* strain were eliminated from the co-culture over time in the presence of fluconazole, but not in the absence of fluconazole (p < 0.05) (Fig. 2A). Similar results were also observed when ρ0 cells of the *gat3Δ::bleMX6* strain were co-cultivated with those of *ume6Δ::KanMX* strain in the presence and absence of 100 μg/mL fluconazole, indicating that selection marker genes do not affect these changes in survival rate (data not shown). Furthermore, similar results were obtained when ρ0 cells of the *gat3Δ* strain were co-cultivated with those of *ume6Δ* strain in the presence and absence of 0.5 μg/mL cycloheximide (data not shown). Rather than using the *gat3Δ* strain, ρ0 cells of the *ash1Δ::bleMX6* strain were co-cultivated with those of *ume6Δ::KanMX* in the presence and absence of 0.5 μg/mL cycloheximide. The survival rate of each strain in the co-culture was estimated in the same way. Consequently, ρ0 cells in the *ume6Δ::KanMX* strain were eliminated earlier from the co-culture over time in the presence of cycloheximide compared to that in the

**Fig. 1** *UME6*, but not *ASH1*, is responsible for the PDR of ρ0 *S. cerevisiae*. Fluconazole or cycloheximide resistance of ρ0 cells in the wild-type strain (FY1679-28C) and its derivative strains, *ume6Δ::bleMX6, ash1Δ::bleMX6, pdr3Δ::bleMX6, and rpdr3Δ::bleMX6* and *gat3Δ::bleMX6* was determined by the spot dilution assay.
cells of the wild-type and ash1Δ mutant strains (p > 0.05) (Fig. 3B and Table S2). Therefore, UME6 and RPD3 are responsible for the enhanced transcriptional expression of PDR5 by Pdr3-mediated retrograde signalling in ρ0 cells but not for basal expression of PDR5 in ρ+ cells. In addition, ρ0 cells, but not ρ+ cells, in the ume6Δ mutant strain, had slightly more reduced PDR3 mRNA levels than those in the wild-type strain, suggesting minor involvement of UME6 in the activation of autoregulated transcriptional expression of PDR3 by retrograde signalling (Figs. 3A and 4).

Furthermore, this study investigated whether the activated transcriptional expression of PDR5 following cycloheximide exposure occurs in ρ0 cells of the ume6Δ mutant strain using real-time RT-PCR. PDR3 and PDR5 mRNA levels in ρ0 cells of the ume6Δ strain were lower than those in the wild-type strain, independent of the addition of cycloheximide. However, PDR5 mRNA levels in ρ0 cells of the wild-type and ume6Δ strains increased 1.6- and 1.55 times, respectively, after exposure to 0.2 μg/mL cycloheximide for 45 min (Fig. 4 and Table S3). These increases in PDR5 mRNA levels in ρ0 cells were statistically significant in the wild-type (p < 0.05), but not in the ume6Δ strains (p > 0.05). This suggests that UME6 is involved in the transcriptional expression of PDR5 in cycloheximide-exposed and unexposed ρ0 cells and the intact induction of PDR5 transcription after drug exposure in ρ0 cells.
Discussion
This study revealed that UME6 and RPD3, but not ASH1, are responsible for enhancing PDR5 expression and PDR by retrograde signalling in ρ⁰ cells of S. cerevisiae. In addition, UME6 was involved in the transcriptional expression of PDR5 in cycloheximide-exposed and unexposed ρ⁰ cells, and the enhancement of PDR5 transcription after cycloheximide exposure was also impaired in ρ⁰ cells of the ume6Δ mutant strain. Reduced PDR5 mRNA levels in the presence and absence of cycloheximide were also reported in ρ⁺ cells of the rpd3Δ strain by Borecka-Melkusova et al.; however, this report was invalidated later by Robbins et al. [17, 19]. Histone deacetylation leads to transcriptional repression and activation [14, 22]. Thus, Ume6 and Rpd3 may serve as enhancers of PDR5 expression by retrograde signalling in ρ⁰ cells, different from their usual roles as repressors.

It is currently unknown how Rpd3 and Ume6 enhance the transcriptional expression of PDR5 and PDR in ρ⁰ cells and why they do not affect basal PDR5 expression in ρ⁺ cells. As Ume6 binds to the PDR5 promoter region in ρ⁺ cells, it may also be localised at the PDR5 promoter region in ρ⁰ cells [23]. If this is true, Rpd3 and Ume6 may directly mediate the activation of PDR5 expression.
by chromatin remodelling and facilitating Pdr3 binding. Furthermore, the enhanced PDR5 transcription and PDR by Rpd3 and Ume6 in p0 cells may be indirectly caused by changes in the expression of other genes. Pdr1 and Pdr3 can bind to the KIX domain of the transcriptional Mediator subunit Med15/Gal11, which mediates sequence-specific transcriptional regulatory proteins and the RNA polymerase II machinery [24]. L-Mediator (of the Mediator complexes) contains the Cdk8 subcomplex, which consists of the cyclin-dependent kinase Cdk8 (Med15/Srb8), Med12 (Srb8), and Med13 (Srb9). Deletion of Med12 from the Cdk8 complex completely suppressed the induction of PDR5 expression in p0 cells but not in p+ cells, indicating a difference in the regulatory machinery of PDR5 transcription between p+ and p0 cells [25]. This difference may be associated with the difference in transcriptional regulation of PDR5 by Rpd3 and Ume6 between p0 and p+ cells.

This study showed that p0 cells in the ume6Δ mutant were less susceptible to fluconazole and cycloheximide than in the pdr3Δ and rpd3Δ mutants as assessed by the spot dilution assay (Fig. 1). This suggests that p0 cells in the ume6Δ mutant, but not the rpd3Δ mutant, maintain Hsp90-dependent antifungal drug resistance and intact ER to Golgi trafficking of Pdr5 [19, 20]. Furthermore, fewer multidrug resistance genes other than PDR5 are downregulated in p0 cells of the ume6Δ mutant than in those of the pdr3Δ mutant [26].

p0 cells, but not p+ cells, in the ume6Δ mutant strain had slightly lower PDR3 mRNA levels than those in the wild-type strain (Figs. 3 and 4). Ume6 binds to the PDR3 promoter region in p+ cells; therefore, it may also bind to the PDR3 promoter region in p0 cells and may directly activate the transcriptionally auto regulated loop of PDR3 by chromatin remodelling and facilitating Pdr3 binding [23].

The emergence of multidrug-resistant fungi is a serious clinical concern [27]. Therefore, the efficacy of combined antifungal agents against multidrug-resistant fungi has been examined [28]. Furthermore, to treat multidrug-resistant fungal infections, the efficacy of using histone deacetylase inhibitors or essential oils from plants in combination with the primary classes of antifungals has also been examined [29–35]. For example, a histone deacetylase inhibitor, trichostatin A, decreases the upregulation of CDR1, ERG1, and ERG11 by azole and enhances azole sensitivity in C. albicans [33]. Uracil-based histone deacetylase inhibitors 1c and 1d reduce acquired resistance to antifungals and trailing growth in C. albicans [34]. In addition, RPD3 is responsible for azole resistance and basal transcription of efflux genes such as CDR1, CDR2, and MDR1 in p+ cells of pathogenic C. albicans [35]. Thus, Ume6 may also be responsible for multidrug resistance via transcriptional regulation of the efflux genes in p+ and p0 cells of pathogenic Candida species. Therefore, identifying specific inhibitors of Ume6 may lead to the development of drugs against multidrug-resistant pathogenic Candida species.

**Conclusions**

PDR5 expression or PDR in the rpd3Δ, ume6Δ, or ash1Δ mutant strains has been examined primarily in p+ cells, but not in p0 cells. In this study, we investigated the roles of RPD3, UME6, and ASH1 in the activation of PDR5 transcription and PDR by retrograde signalling in p0 cells. Using spot dilution and co-cultivation assays, we
have shown that RPD3 and UME6, but not ASH1, contribute to the PDR in \( p^0 \) cells of \( S.\) cerevisiae. In addition, using real-time PCR assay, we have shown that RPD3 and UME6, but not ASH1, are involved in the transcriptional expression of PDR5 in \( p^0 \) cells, and UME6 also contributes to PDR5 transcription and its enhancement in cycloheximide-exposed \( p^0 \) cells. This work provides useful knowledge on the genetic basis of yeast multidrug resistance via transcriptional regulation of efflux genes.

**Methods**

**Yeast strains and media**

FY1679-28C (MATa, ura3–52, leu2-D1, trp1-D63, his3-D200, GAL2+) and W303–1A (MATa, ura3–1, leu2–3,112, trp1–1, his3–11,15, ade2–1, can1–100, rad5–535) strains were used as wild-type strains [27]. W303–1A was provided by the National Bio-Resource Project, Japan. To construct the gene deletion mutant strains, open reading frames of UME6, PDR3, RPD3, ASH1, or GAT3 were replaced with KanMX or bleMX6 gene cassettes by PCR-mediated one-step gene disruption in the FY1679-28C or W303–1A background [36].

The strains described above were grown on glycerol-rich YPG agar plates (2% glycerol, 1% yeast extract, 2% bactopeptone, 2% agar) to eliminate \( p^0 \) cells and obtain \( p^+ \) cells for real-time RT-PCR [17]. The \( p^0 \) derivatives of the strains described above were obtained by plating the cells twice on YPD agar plates (2% glucose, 1% yeast extract, 2% bactopeptone, and 2% agar) containing 40 \( \mu \)g/ml ethidium bromide [26]. Yeast cells were grown in YPD medium (2% glucose, 1% yeast extract, and 2% bactopeptone) at 30°C with shaking.

**Spot dilution assay**

A spot dilution assay was conducted to estimate the relative resistance of each yeast strain to fluconazole or cycloheximide [37, 38]. Three independently derived \( p^0 \) cells from each yeast strain were aerobically grown to an OD\(_{600}\) of 0.6–0.9 at 30°C in YPD medium. Five microliters of 10-fold serial dilutions of the logarithmic phase cultures containing the same number of cells were spotted on YPD plates containing or not containing 20 \( \mu \)g/ml fluconazole (Nacalai Tesque) (or 0.5 \( \mu \)g/ml cycloheximide) and incubated at 30°C for 7 days. Representative plate images of three replicates were captured after culturing at 30°C for 7 days.

**Co-cultivation of two gene deletion mutants replaced with KanMX or bleMX6 gene cassettes**

The \( \text{ume6}\Delta::\text{KanMX} \) and \( \text{ash1}\Delta::\text{bleMX6} \) mutant strains, or the \( \text{ume6}\Delta::\text{bleMX6} \) and \( \text{ash1}\Delta::\text{KanMX} \) mutant strains were co-cultivated in YPD medium containing or not containing 100 \( \mu \)g/mL fluconazole (or 0.5 \( \mu \)g/ml cycloheximide). The aliquots of the co-culture were recovered immediately before adding drugs and at various times after addition of the drugs, and spread on the YPD plates containing G418 (Wako) or Zeocin (Nacalai Tesque) [20]. The viability of each strain at each time point was estimated from the colony numbers on G418 and Zeocin plates [20]. The previous experiments were also performed in the \( \text{ume6}\Delta::\text{KanMX} \) and \( \text{gat3}\Delta::\text{bleMX6} \) mutant strains, or the \( \text{ume6}\Delta::\text{bleMX6} \) and \( \text{gat3}\Delta::\text{KanMX} \) mutant strains.

**RNA extraction from \( p^0 \) and \( p^+ \) cells of each mutant strain grown to the logarithmic phase**

Two independently derived \( p^0 \) and \( p^+ \) cells from each yeast strain were grown to an OD\(_{600}\) of 7–9 in YPD, diluted to an OD\(_{600}\) of 0.2, and grown for an additional 5 h in duplicate [39, 40]. Aliquots of the duplicates were recovered. The cells in the aliquots above were pelleted, washed, frozen at −80°C, and used to extract total RNA [39, 40]. Total RNA was isolated from yeast cells using Nucleospin RNA Plus (TaKaRa), according to the manufacturer’s protocol.

**RNA extraction from \( p^0 \) cells exposed to drug**

Independently derived \( p^0 \) cells from each yeast strain were grown to an OD\(_{600}\) of 7–9 in YPD, diluted to an OD\(_{600}\) of 0.2, and grown for an additional 5 h in duplicate [39, 40]. Aliquots of the triplicates were harvested just before the addition of cycloheximide to the medium. Cycloheximide (0.2 \( \mu \)g/mL) was added to one of the triplicates, and the triplicates were grown for 45 min and 90 min at 30°C. Aliquots of the triplicates were recovered at 45 min and 90 min after adding cycloheximide to one of the triplicates. The cells in the aliquots above were pelleted, washed, frozen at −80°C, and used to extract total RNA [39, 40]. Total RNA was isolated from yeast cells before and after exposure to cycloheximide using Nucleospin RNA Plus (TaKaRa), according to the manufacturer’s protocol.

**Real-time RT-PCR**

Reverse transcription of total RNA was performed using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa). SYBR Green qRT-PCR was performed using the TB Green® Premix Ex Taq II (TaKaRa) in a Step One Real-time PCR system (Applied Biosystems). For quantitative PCR analysis, the housekeeping gene ACT1 was used as an endogenous control to normalise the expression level of each target gene [41]. Minus reverse transcriptase control was used as the negative control. qPCR for each sample was performed in duplicate or triplicate. Serial dilutions of the control cDNA from the wild-type
strain were prepared to produce a standard curve for each primer pair. The primers used for PDR3 were: forward, 5'-TACCGCAGGAGGATATGTTCCCA-3' and reverse, 5'-GCTTATCGCAGTGGCAGATGCT GTAC-3', yielding a PCR product of 117 bp. The qPCR for PDR5 was performed using primers 5'-CTCTGAGAG AACCCTGAACAAAGATATGCTA-3' (forward) and 5'-ATAAGCTTACGGGTTCGTTACGT-3' (reverse) to amplify a fragment of 165bp. The primers used for ACT1 were as follows: forward, 5'-CAAATTATGTTT GAAACTTTCACTTCCAG-3' and reverse, 5'-ACGTAGTAAACACCATCACCGGAATC-3', yielding a PCR product of 125bp.

Statistical analysis
The survival rate of ume6Δ strain at each time point in Fig. 2 was normalized by that at 0h. Paired t-test was used for statistical analysis in Figs. 2 and 4. Unpaired Student’s t-test was used for statistical analysis in Fig. 3. p < 0.05 was considered significant.

Abbreviations
ABC: ATP-binding cassette; PDR: Pleiotropic drug resistance; ER: Endoplasmic reticulum; PDRE: Pleiotropic drug response elements.

Supplementary Information
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Author’s contributions
YY performed acquisition, analysis and interpretation of data. YY made contributions to conception, design and drafting of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are within the manuscript and the Additional files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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