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Analysis of the regulatory effect of ACTG2 on biological behavior of bladder cancer cells based on database screening

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ABSTRACT

To identify genes aberrantly expressed in bladder cancer (BC) in the GEO dataset GSE 52519, and analyze the impact of abnormal Actin Gamma 2, Smooth Muscle (ACTG2) expression on BC cells. GSE 52519, a public dataset in the Gene expression omnibus (GEO) database, was selected for differential expression analysis. Differentially expressed ACTG2 vectors were selected to construct the aberrant expression vectors and transfected into BC T24 and J82 cells. The influences of ACTG2 on BC cell biological behavior were determined by cell cloning, Transwell and flow cytometry, and alterations in cell cycle status were observed. A total of 166 DEGs were found in the GSE 52519 dataset, among which ACTG2 was abnormally low in expression. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses identified that the keywords involved mainly included “extracellular region”, “cytoskeleton”, “Vascular smooth muscle contraction”, “IL-17 signaling pathway”, etc. Further, through online data analysis, ACTG2 was also found to be under-expressed in neoplastic diseases such as bladder urothelial carcinoma (BLCA) and prostate adenocarcinoma (PRAD). *In vitro*, ACTG2 presented lower expression in T24 and J82 than in SV-HUC-1 ($P<0.05$). Enhanced capacities to proliferate and invade and reduced apoptosis of T24 and J82 were found after silencing ACTG2 expression, with shortened G0-G1 phase and prolonged S phase ($P<0.05$). However, overexpressing ACTG2 came with decreased BC cell activity, enhanced apoptosis, as well as prolonged G0-G1 phase and shortened S phase ($P<0.05$). In conclusion, low expression of ACTG2 in BC can shorten the G0-G1 phase of BC cells, prolong the S-phase.

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Introduction

Bladder cancer (BC), a neoplastic disease with a very high incidence in males, is one of the most common malignancies in the world at present (1). According to statistics, the disease shows an ever-high incidence, with the global cases newly diagnosed with BC exceeding 800, 000 in the year 2020 (2). In clinical practice, various causes such as smoking, living environment, and urinary tract diseases are considered as risk factors for BC (3). BC usually has no specific symptoms at the beginning of the disease, but as the disease progresses, hematuria, painful urination, and upper urinary tract obstruction may occur, and in severe cases, infection and necrosis of the entire urinary system may cause organ failure and endanger the patient's life (4). Surgery remains the mainstay treatment for BC; it has ideal therapeutic effects for early-stage BC, but cannot bring a cure to middle and late-stage patients (5). As a result, the prognosis of BC is extremely unsatisfactory, with a 5-year overall survival rate below 40% in advanced BC patients (6). Thus, the clinic is also trying to find more effective treatments for BC.

Molecular pathogenic research has gradually become the focus of clinical research in recent years, as research has deepened. Among them, protein-coding genes (PCGs) are one of the genetic materials in the human body that are critical in modulating the biological behavior of cells and tissues, with their involvement in the development of many diseases demonstrated . For BC, the research on PCGs is also a hot spot in modern clinical research. For example, Nectin-4 has been proven to promote BC infiltration (7), and Cluster of Differentiation 10 (CD10) is highly linked to BC histopathological grading (8). It is precise because of the important role of PCGs that it is clinically believed that they may be a breakthrough in future diagnosis and treatment of BC (9). Through online database screening, we found that Actin Gamma 2, Smooth Muscle (ACTG2) is a gene closely associated with smooth muscle cells and has been confirmed to participate in the development of congenital bladder dilatation (10, 11). Meanwhile, a strong connection between ACTG2 and progesterone in the reproductive system and semen quality has been established (12, 13), but its role in BC remains poorly characterized. And in colorectal cancer, liver cancer and other tumor diseases, ACTG2 is also considered to be an important tumor influencing factor with great significance (14, 15).

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This shows that there may be an extremely close potential link between ACTG2 and malignant tumors.

We speculate that ACTG2 may be of great significance to the occurrence and development of BC. Consequently, this paper will analyze the role played by ACTG2 in BC, to assist the clinical understanding of the pathogenesis of BC and provide new research directions for future BC diagnosis and management.

Materials and Methods

The Gene expression omnibus (GEO) database (URL: https://www.ncbi.nlm.nih.gov/geo/) was searched for BC with the organism selected as "homo sapiens". After review, the publicly available dataset, GSE 52519, was finally selected for GEO2R analysis (GPL 6884), including 12 groups of samples (9 groups of BC tissues and 3 groups of healthy bladder tissues). The absolute values of Log (FC) >2 and P<0.05 were used to filter the results, and a volcano plot and a heat map were plotted.

Protein-protein interaction (PPI) networks

PPI network analysis was carried out using differentially expressed genes (DEGs) in the STRING database (URL: https://string-db.org/), and the relationship between these DEGs was observed.

Enrichment analysis

Cluster analysis was carried out on the DEGs through the pre-built gene annotation databases [Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)], and the keywords involving the most genes were obtained.

Online gene expression analysis

ACTG2 expression in BC and other neoplastic diseases was analyzed using GEPIA (URL: http://gepia.cancer-pku.cn/index.html) and ENCORI (URL: https://starbase.sysu.edu.cn/) databases.

Cell data

Human bladder transitional cell carcinoma T24 and J82 cells, as well as human normal urothelial SV-HUC-1 cells, all ordered from ATCC, were cultivated in a 10% fetal bovine serum (FBS)-supplemented medium in a 37 ℃ and 5% CO_2 incubator.

Polymerase Chain Reaction (PCR) detection

The total RNA of the tested cells extracted by TRIzol (ThermoFisher Scientific, USA) was reverse transcribed to cDNA (SuperScript IV CellsDirect cDNA Synthesis Kit, ThermoFisher Scientific, USA), after which 20 μg were taken for amplification under the reaction conditions of 95 ℃ for the 30 s, 95 ℃ for 5 s, 65 ℃ for 30 s and 72 ℃ for 30 s, for 40 cycles. ACTG2 expression by PCR instrument (Preflex PCR instrument, ThermoFisher Scientific, USA), normalized against GAPDH, was computed by $2^{-\Delta\Delta CT}$. The primer sequences were designed and synthesized by Suzhou GENEWIZ Biotechnology Co.

Transfection of cells

The aberrant expression vectors were commissioned to be designed and constructed by Jiangsu Hillgene Biotech Co., Ltd. According to the Lipofectamine 2000 transfection kit recommendations, vectors that can target to increase ACTG2 (ACTG2-el) and silence ACTG2 (ACTG2-si), as well as the empty vector (ACTG2-ev), were transfected into T24 and J82, respectively. PCR verified the transfection success rate with the same method as described above.

Cell proliferation assay

Another group of cells was adjusted to 200 cells/mL and inoculated in 6-well plates (5×10^6 cells/well) for 5 days of constant temperature culture, after which 500 μL FBS was added. The supernatant was discarded after visible colony formation, and the residue was subjected to 4 % paraformaldehyde immobilization and 0.1 % crystal violet staining, for microscopical counting of the cells generated.

Cell invasion assay

5×10^4 cells/mL were inoculated in the apical chamber coated with Matrigel glue, and the 10 % FBS-supplemented cell culture solution was placed into the basolateral chamber. After constant temperature culture for 24 h and removal of the suspended cells on the surface layer, cells were immobilized and dyed to microscopically count the membrane-penetrating cells under five randomly selected visual fields.

Apoptosis detection

The PBS-rinsed cells were resuspended with 100 μL binding buffer, added with AnnexinV and PI (Abcam, USA) in turn, and incubated in the dark for 15 min. The apoptosis rate was detected by flow cytometry. In addition, DNA cell cycle analysis software was utilized to analyze alterations in cell life cycle status: G0/G1 phase: DNA replication has not yet begun, and is also the least amount of DNA (the first peak in the flow test result graph). s phase: DNA replication begins, to the completion of replication, a process of doubling DNA to doubling DNA (the middle span in the flow test result graph). g2 phase: a period of time between the completion of DNA replication and division, when the cell contains doubled DNA (the third peak in the flow test result graph). m phase: a period of time between the completion of DNA replication and division, when the cell contains doubled DNA (the third peak in the flow test result graph). M phase: the process of cell division, when the cells also contain diploid DNA, which cannot be separated from G2 phase by the method of DNA content. Another group of cells were subjected to lysis with a RIPA buffer (Abcam, USA), followed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation, Polyvinylidene Fluoride (PVDF) membrane transfer, and sealing with 5% defatted milk for

Table 1. The used primer names and sequences.

	F (5'-3')	R (5'-3')
ACTG2	GCGT GTAGCACCTGAAGAG	GAATGGCGACGTACATGGCA
GAPDH	CGGACCAATACGACCAAATCCG	AGCCACATCGCTCAGACACC

2 h. After washing with primary antibodies B-cell lymphoma-2 associated X (Bax) (1:1000), cl-Caspase-3 (1:1000) and β-actin (1:1000) the membrane was incubated at 4°C overnight. It was grown with a second antibody (1:5000) for 2 h after rinsing the next day and developed for the gray-value analysis of the target bands by ImageJ. Protein antibodies were purchased from Abcam, USA.

Statistical methods

This study employed SPSS 21.0 software for statistical analysis. All experiments in this study were done in triplicates, with the results indicated by ($\bar{\chi}\pm s$); for inter-group and multi-group comparisons, the independent samples t test and variance analysis plus Least-SignificantDifference (LSD) post-hoc test were used, respectively, with the presence of statistical significance indicated by $P<0.05$.

Results

Database screening results

In the GSE 52519 dataset, a total of 166 PCGs were found, among which Purkinje Cell Protein 4 (PCP4), Desmin (DES), Actin Alpha Cardiac Muscle 1 (AC1TC1), Myosin Heavy Chain 11 (MYH11), etc. were the genes with the most significant differential expression (Fig 1A, B). The PPI network analysis revealed some potential relationships among most DEGs but little correlation of Stonin 1 (STON1), Integral Membrane Protein 2A (ITM2A), Calpain 8 (CAPN8), Chloride Channel Accessory 4 (CLCA4), etc. with BC (Fig 1C).

Enrichment analysis

GO analyses showed that the DEGs in the GSE 52519 dataset mainly involved keywords such as "extracellular region", "cytoskeleton", "supramolecular fiber", etc. (Fig 2A). KEGG analysis showed that the key words of differential genes were: "Vascular smooth muscle contraction", "Interleukin 17 (IL-17) signaling pathway", "Transcriptional misregulation in cancer", etc (Fig 2B). Among them, ACTG2 was low expressed in BC, with a strong connection with the keyword "actin cytoskeleton", "extracellular region ", "Vascular smooth muscle contraction", etc.

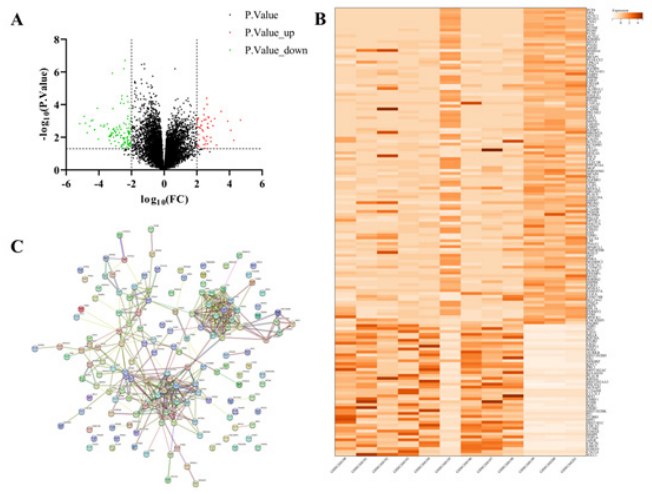

Figure 1. Database screening results. A: Volcano plot of differentially expressed genes (DEGs) in the GSE 52519 dataset. B: Heat map of DEGs in the GSE 52519 dataset. C: PPI network of DEGs.

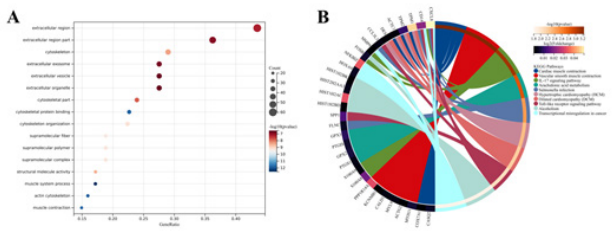

Figure 2. Enrichment analysis. A: GO analysis of differentially expressed genes in the GSE 52519 dataset. B: KEGG analysis of differentially expressed genes in the GSE 52519 dataset.

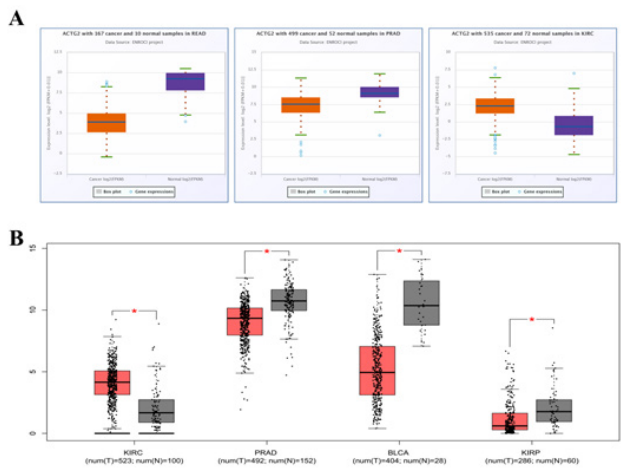

Figure 3. Online database analysis of ACTG2 expression. A: ACTG2 expression in the ENCORI database. B: ACTG2 expression in the GEPIA database.

Online database analysis of ACTG2 expression

The analysis of ACTG2 expression in the ENCORI database showed that it was also down-expressed in bladder urothelial carcinoma (BLCA) and prostate adenocarcinoma (PRAD) ($P<0.05$), but was over-expressed in kidney renal clear cell carcinoma (KIRC) ($P<0.05$, Fig 3A). GEPIA database analysis also revealed under-expressed ACTG2 in BLCA, PRAD and kidney renal papillary cell carcinoma (KIRP) while up-regulated ACTG2 in KIRC ($P<0.05$, Fig 3B). This implies that ACTG2 is lowly expressed in several malignancies.

ACTG2 expression in BC cells

ACTG2 expression levels in T24 and J82 were (1.12±0.22) and (1.18±0.15), respectively, lower than those in SV-HUC-1 ($P<0.05$, Fig 4), confirming the low

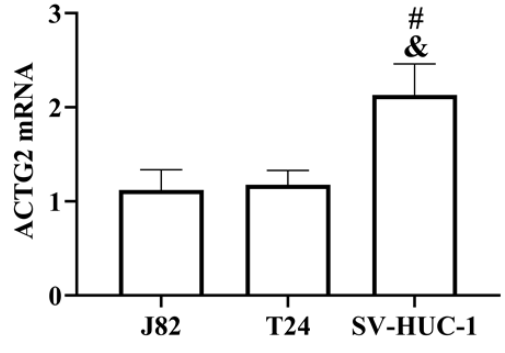

Figure 4. ACTG2 expression in BC cells.# represents a comparison with J82, & represents a comparison with T24.

expression of ACTG2 in BC.

Impacts of ACTG2 on BC cell activity

ACTG2 expression was higher in the ACTG2-el group than in the other two groups and was even lower in the ACTG2-si group as compared to the ACTG2-ev group (P<0.05, Fig 5A). In the cell cloning experiment, the ACTG2-si group also showed a higher cell cloning rate than the other two groups, and the cell cloning rate was lower in the ACTG2-el group versus the ACTG2-ev group (P<0.05, Fig 5B). Finally, in the Transwell assay, the number of membrane-penetrating cells was evidently increased in the ACTG2-si group and markedly decreased in the ACTG2-el group (P<0.05, Fig 5C). This suggests that elevated ACTG2 expression can inhibit the growth of BC cells.

Impacts of ACTG2 on BC cell apoptosis

The apoptosis rate was the lowest in the ACTG2-si group and the highest in the ACTG2-el group among the three groups (P<0.05, Fig 6A). Similarly, the detection of apoptosis protein expression revealed lower Bax and cl-Caspase-3 protein in the ACTG2-si group compared with the other two groups (P<0.05); the ACTG2-el group showed higher Bax and cl-Caspase-3 protein than ACTG2-ev group (P<0.05, Fig 6B). This suggests that elevated ACTG2 expression can promote apoptosis in BC cells.

Influence of ACTG2 on BC cell cycle status

Finally, the BC cell cycle changes were observed, and no significant changes occurred in the G2-M phase of three groups of cells (P>0.05), while the G0-G1 phase of the ACTG2-si group was significantly shortened and the S-phase was prolonged compared with ACTG2-el and ACTG2-ev groups (P<0.05, Fig 7); and in comparison with ACTG2-ev group, the G0-G1 phase was longer and the S-phase was shorter in ACTG2-el group. This implies that inhibition of ACTG2 expression can prolong the life cycle of BC cells.

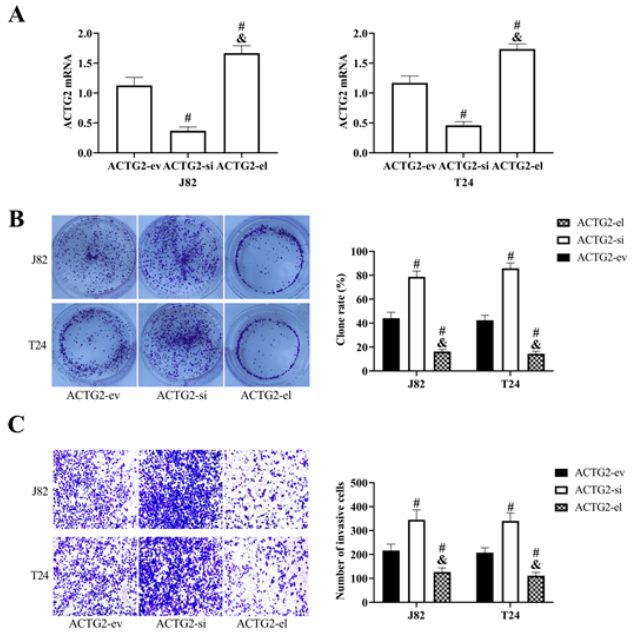

Figure 5. Impacts of ACTG2 on BC cell activity. A: ACTG2 expression detected by PCR. B: Influence of ACTG2 on BC cell cloning ability by cell cloning experiment. C: Influence of ACTG2 on BC cell invasion ability by Transwell assay. # represents a comparison with ACTG2-ev goup, & represents a comparison with ACTG2-si group.

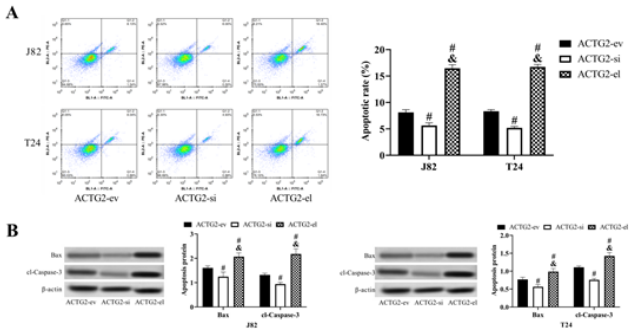

Figure 6. Impacts of ACTG2 on BC cell apoptosis. A: Influence of ACTG2 on BC cell apoptosis rate by flow cytometry. B: Influence of ACTG2 on apoptosis protein expression in BC cells by Western blotting. # represents a comparison with ACTG2-ev goup, & represents a comparison with ACTG2-si group.

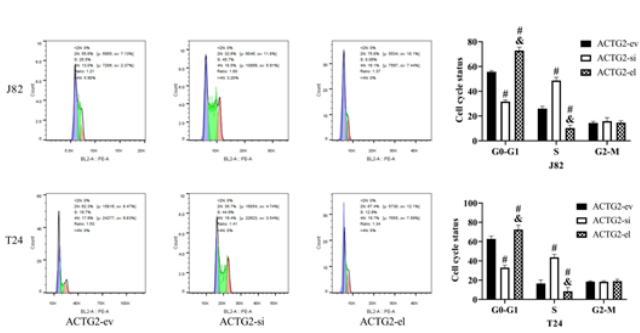

Figure 7. Influence of ACTG2 on BC cell cycle status by flow cytometry.# represents a comparison with ACTG2-ev group, & represents a comparison with ACTG2-si group.

Discussion

Currently, BC remains a high-incidence malignancy worldwide, seriously threatening the life safety of patients. Finding a new diagnosis and treatment method for BC is just the focus and difficulty of modern clinical research (16). As a hot spot in modern tumor molecular pathogenesis research, PCGs have important regulatory capabilities for cells and tissues on the one hand and can be detected in human blood, body fluids and tissues on the other, with high convenience. Therefore, they have also been hailed as a breakthrough in the future research of neoplastic diseases including BC (17, 18). In this study, we conducted a preliminary screening for DEGs in BC through online databases. ACTG2, as a highly conserved member of the actin family, participates in the movement of almost all cell types and is universally expressed in eukaryotic cells (19). Although the exact expression of ACTG2 in BC is currently unknown, we tentatively infer a possible close association between ACTG2 and BC based on related studies (10-13). Hence, we chose ACTG2 for further research.

First of all, the online database analysis showed generally low expression of ACTG2 in neoplastic diseases, similar to the results of the differential expression analysis described above. Meanwhile, we found through previous studies that ACTG2 was reduced in chronic intestinal obstruction, choledochal cysts and other diseases (20-

22), which agrees with the results of the above-mentioned bioinformatics analysis and can support our results. However, the exact influence of ACTG2 on BC cannot be determined by the above experiments. To further understand and confirm the correlation of ACTG2 with BC, we conducted *in vitro* experiments by purchasing BC cell lines. First, ACTG2 expression was found to be lower in BC T24 and J82 cells compared to SV-HUC-1, which confirms the low expression of ACTG2 in BC and is in agreement with the above experimental results and inference. Subsequently, by constructing abnormal expression vectors of ACTG2 and transfecting them into BC cell lines, we found that the capacities of BC cells to proliferate and invade were weakened and the apoptosis was enhanced after up-regulating ACTG2; whilst BC cell activity increased and apoptosis decreased after silencing ACTG2. This suggests that the low expression of ACTG2 in BC can promote malignant cell growth and inhibit their apoptosis. This is also consistent with previous studies (14, 23), further demonstrating the important role of low ACTG2 expression in the development of neoplastic diseases. Finally, through cell cycle analysis, we can also see that the G0-G1 phase of BC cells was shortened and the S phase was prolonged after silencing ACTG2, while up-regulating ACTG2 contributed to the opposite results, suggesting that low ACTG2 expression can prolong the life cycle of BC cells and contribute to the malignant differentiation of BC, thus aggravating the development of BC. On the other hand, the blocking effect of ACTG2 up-regulation on the S-phase of cells suggests that targeted up-regulation of ACTG2 through molecular targeting may be a new direction for future BC therapies. This is of great significance for the current clinical situation where the therapeutic effect of advanced BC is not ideal. Its clinical application, of course, requires more experimental confirmation.

Although this study conducted a preliminary analysis of the role played by ACTG2 in BC, there are still many shortcomings. For example, clinical cases were not included in this study, so the exact clinical expression of ACTG2 cannot be determined for the time being. Second, we need to further determine the impact of ACTG2 on the actual tumor formation of BC through tumor transplantation experiments in living mice. In the follow-up, we will conduct a more in-depth and comprehensive experimental analysis aiming at addressing the above limitations and clarifying the influencing mechanism of ACTG2 on BC, so as to obtain more reliable experimental results for clinical reference.

Low expression of ACTG2 in BC can shorten the G0-G1 phase of BC cells, prolong the S-phase, and promote their malignant development, which may be a new direction for future BC diagnosis and treatment.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors Contributions

Xue Song and Hailong Liu designed the study and wrote the manuscript. Ping Xiang and Wenhao Miao collected and analyzed data. Haitao Liu and Haibo Shen supervised the research. All authors read and approved the final submitted manuscript.

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Ethics approval and consent to participate

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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