WSTF Modulates Migration of Breast Cancer Through Regulating Expressions of P21 and CCL2

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Background: *The purpose of this study is to investigate the regulation of Williams syndrome transcription factor (WSTF) on the migration ability of breast cancer cells, as well as whether cyclin-dependent kinase inhibitor 1A (p21) and C-C Motif Chemokine Ligand 2 (CCL2) genes are involved in.*

Methods: *Quantitative real-time PCR (qRT-PCR) and Immunohistochemistry (IHC) were used to test gene expression in breast cell lines and tissues, respectively. Western blotting was used to test protein expression. SiRNAs were transfected to knockdown WSTF to verify its functions. Transwell assay was used for migration determination.*

Results: *In our research, we found that WSTF was upregulated in breast cancer cells and tissues, compared to adjacent normal controls. Knockdown of WSTF markedly suppressed migration abilities of MDA-MB-231 and MCF-7 cells. Rescue assays illustrated that overexpression of p21 and CCL2 reversed the inhibition effect of WSTF knockdown on migration ability of breast cancer cells.*

Conclusion: *These findings revealed that hyper-expressed WSTF in breast cancer promoted the cell migration ability by upregulating p21 and CCL2 expressions.*

BACKGROUND

Breast cancer (BC) is a common malignant tumor that occurs in the breast epithelium, which is a primary cause of deaths about cancer around the world.[1] Despite advances in diagnosis and combined therapies, the prognosis of BC patients was still poor.[2,3] Therefore, an understanding of the mechanisms underlying BC development needs to be further studied.

Williams syndrome transcription factor (WSTF) is found in patients with Williams syndrome and is encoded by bromodomain, which is adjacent to zinc finger domain 1B (BAZ1B) gene.[4] It is involved in diverse molecular processes, including transcription, replication, chromatin remodeling and DNA damage response.[5] WSTF activated estrogen receptor signaling to promote the growth of BC cells. [6]

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Our previous study found that WSTF could activate the the oncogenic pathways in colon tumor cells through forming a complex with neuregulin-3 (NRG3). [7] Nevertheless, the function and mechanism of WSTF in BC metastasis remain unclear.

The cyclin-dependent kinase inhibitor 1A (p21), which is encoded by cyclin-dependent kinase inhibitor 1A, could directly inhibit cyclin-dependent kinases in G1, S, and G2 phase in the cell cycle. [8-10] Upregulation of p21 leads to the cell-cycle arrest and invasion of BC cells.[11] The C-C Motif Chemokine Ligand 2 (CCL2) can recruit monocytes and macrophages to sites of inflammation as a chemokine. [12] However, the upstream regulators of p21 and CCL2 in BC need to be further clarified.

In this study, we examined the expression of WSTF in human BC tissues and cell lines. Then we analyzed the effect of WSTF on the migration characteristics and the regulation of WSTF on the expressions of p21 and CCL2 in BC cells. Furthermore, we clarified whether the function of WSTF in regulating migration was involved by p21 and CCL2. It could provide a basis for the mechanism of BC.

MATERIALS AND METHODS

Patients and Samples

The BC tissues and the corresponding adjacent normal tissues were collected (n=30) form patients (aged 25-60 years) were obtained from Tangshan People's Hospital (Tangshan, China). All the patients were not treated with chemotherapy, radiotherapy or other therapies before surgery. This study was approved by the Ethics Committee of Tangshan People's Hospital. Informed consent was signed by all patients. Experiments involving human tissues were conducted in accordance with the Declaration of Helsinki.

Immunohistochemical Analysis

Paraffin tissue sections of breast cancer and adjacent tissues were collected. After dewaxing, hydration, blocking with hydrogen peroxide, antigen retrieval and blocking at room temperature, the samples were incubated with WSTF antibody (1:200 dilution, Abcam, USA) overnight at 4°C. The slides were stained with secondary antibody (Abcam, USA) incubation after overnight, then counterstained with hematoxylin (Leagene, Beijing, China). Neutral resin sealed the slides and observed under microscope after drying.

Cell culture

The MDA-MB-231, MCF-7 and MCF-10A, were purchased from the Basic Medical Cell Center, Institute of Basic Medicine, Chinese Union Medical University (Beijing, China). MDA-MB-231 cell line was maintained in RPMI-1640 medium containing 15% fetal bovine serum (HyClone, USA) in a humidified incubator at 37°C under 5% CO2. MCF-7 cell lines was maintained in DMEM medium containing 15% fetal bovine serum (HyClone, USA) in a humidified incubator at 37°C under 5% CO2. MCF-10A cell line was maintained in special medium for MCF-10A (Procell, China) in a humidified incubator at 37°C under 5% CO_2.

Cell Transfection

Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. WSTF siRNAs and control siRNAs were purchased from GenePharma (Suzhou, China). pEGFP-N1/p21 plasmids were purchased from Generay (Shanghai, China). pEGFP-N1 plasmids were purchased from Promega (Wisconsin, USA). pEGFP-N1/ CCL2 plasmids were purchased from generay (Shanghai, China).

qRT-PCR

Total RNAs were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription using cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA).

RT-qPCR analysis was performed using SYBR® Premix Ex TaqTM II(Tli RNaseH Plus, Takara, Japan).

The thermo cycler programs were as follows: 95°C for 3mins and 40 cycles of 95°C for 30s, 60°C annealing temperature for primer pairs for 30s, and 72°C for 30s, using GAPDH as an internal reference. The results were evaluated using the $2^{-\Delta\Delta CT}$ method. Each reaction was performed in triplicate.

Western Blot

MDA-MB-231 and MCF-7 samples were harvested and extracted using RIPA lysis buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol), and equal amounts of cells extracts were separated on 10% SDS-PAGE gels and then transferred the separated protein samples onto a polyvinylidene fluoride (PVDF) membrane (iBlot, Shanghai, China). After blocking with 5% Blotto for 2h, the membrane was incubated at 4℃ overnight with the primary antibody, WSTF，p21，CCL2 (1:500, Abcam, USA) and GAPDH (1:2000, Saierbio, Tianjin, China). HRP mouse IgG secondary antibodies (ZSGB-BIO, Beijing, China) were added and incubated for 1.5h at room temperature. The membranes were washed four times for 5mins with TBST buffer, then put the membranes into the Chemiluminescence Reagent (Bohai, Hebei, China) for 30s, which assessed protein expression. Finally, blot signals were visualized by chemiluminescent gel imaging analysis system to quantify the band intensities.

Transwell Assay

The migratory capability of cells was determined using a Transwell chamber culture system (8μm pore; Corning, Cambridge, MA). Briefly, 3×104 per well of MDA-MB-231 (3×104 per well of MCF-7) cells were resuspended in culture medium without FBS and seeded in the upper chamber. Then the chamber was placed into a 24-well plate containing 600μl of culture media with 20% FBS. The upper chamber coated without Matrigel incubated at 37°C for 24h. After the cultured time, the cells were fixed with 33% (v/v) acetic acid (glacial acetic acid: methyl alcohol is 1:3) and stained with 0.1% crystal violet (Solarbio, Beijing, China) for 20mins. Cells on the upper membrane were carefully removed with a cotton stick. The number of migrated cells per field was counted with an Olympus IX 71 (Tokyo, Japan).

Statistical analysis

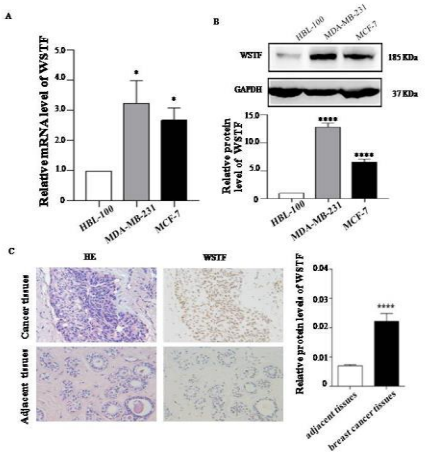
All statistical analyses were performed using SPSS 17.0 software (IBM, USA). Each experiment was carried out at least in triplicate and all results were presented as the mean ± SD. Student's *t*-test and variance test were used to assess statistical significance between groups. $^{*}P<0.05$ was considered statistically significant difference.

RESULTS

WSTF was Upregulated in BC Cells and Tissues

The mRNA and protein levels of WSTF in BC cells and tissues were detected by RT-qPCR and western blot assay. The mRNA levels of WSTF in MDA-MB-231 and MCF-7 cells were all significantly higher than that in MCF-10A cells ($P<0.05$, $P<0.05$) (Figure 1A). The protein levels of WSTF in MDA-MB-231 and MCF-7 cells were significantly higher than that in MCF-10A cells ($P<0.001$, $P<0.001$, Figure 1B). The WSTF was hyper-expressed in cancer tissues compared to those of adjacent normal tissues (n=30) (Figure 1C). These results suggested that WSTF probably functioned as an oncogene in BC.

Figure 1 The expressions of WSTF in breast cancer cells and tissues



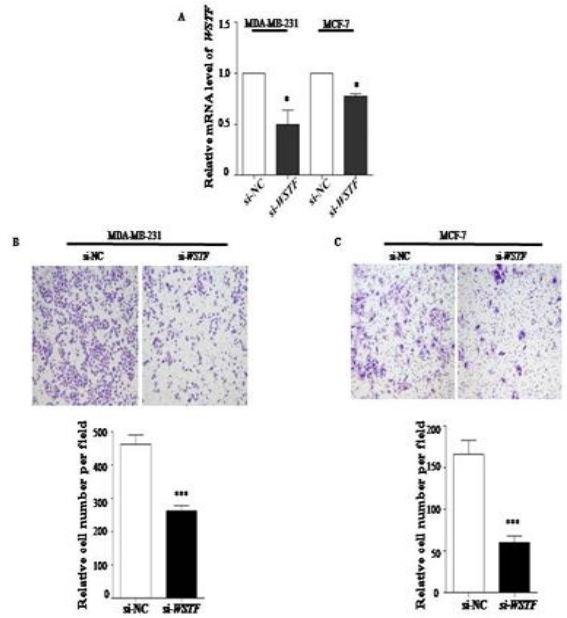
Note: (A): The mRNA levels of WSTF in normal breast epithelial cell HBL-100, breast cancer cell MDA-MB-231 and MCF-7 were detected by RT-qPCR, using GAPDH as internal reference. (B): The protein levels of WSTF in HBL-100, MDA-MB-231 and MCF-7 cells were detected by western blot assay, using GAPDH as internal reference. (C): Representative results of HE and immunohistochemical staining of WSTF (Williams syndrome transcription factor) in breast cancer and adjacent tissues (× 400).

The semi-quantification of the immunohistochemical results of WSTF. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, * P<0.05, **** P<0.001.

Knockdown of WSTF Suppressed Migration Abilities of MDA-MB-231 and MCF-7 Cells

We then analyzed the effect of knockdown of WSTF on migration abilities of MDA-MB-231 and MCF-7 cells. The specific small interference RNA targeting WSTF (si-WSTF) and negative control siRNA (si-NC) were separately transfected into MDA-MB-231 and MCF-7 cells. The RT-qPCR results showed that the mRNA levels of WSTF in the si-WSTF MDA-MB-231 and si-WSTF MCF-7 cells were all significantly reduced compared to the si-NC cells at 24h post-transfection (P<0.05, P<0.05, Figure 2A). The western blot assay showed that the protein levels of WSTF in the si-WSTF MDA-MB-231 and si-WSTF MCF-7 cells were all significantly reduced compared to the si-NC cells at 48h post-transfection (Figure 2B). The above results proved that si-WSTF could effectively reduce the level of cell endogenous WSTF. Transwell assay showed that si-WSTF markedly inhibited the migration abilities of MDA-MB-231 (P<0.001, Figure 2C) and MCF-7 cells (P<0.001, Figure 2D). These suggested that WSTF positively regulated migration abilities of MDA-MB-231 and MCF-7 cells.

Figure 2: Effects of WSTF knockdown on migration abilities of MDA-MB-231 and MCF-7 cells

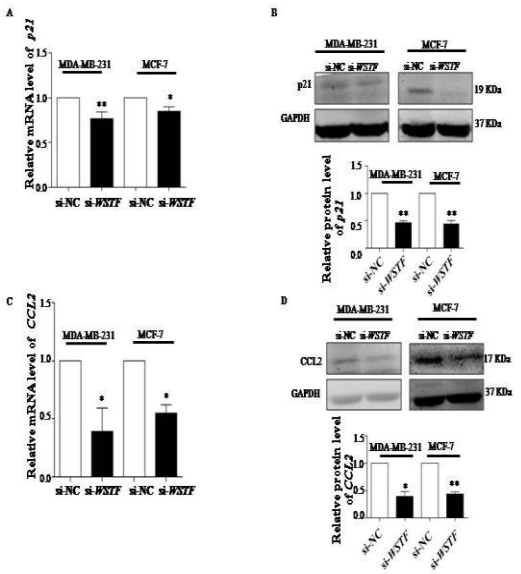


Note: MDA-MB-231 and MCF-7 cells were transfected with siRNAs for WSTF (si-WSTF) and negative control (si-NC), respectively. (A): The mRNA levels of WSTF in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. (B): Migration ability of each group MDA-MB-231 cells was detected by transwell assay. (C): Migration ability of each group MCF-7 cells was detected by transwell assay. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, * P<0.05, *** P<0.005.

Knockdown of WSTF Suppressed Expressions of P21 and CCL2 in BC Cells

The RT-qPCR results showed that the mRNA levels of P21 in si-WSTF MDA-MB-231 cells and MCF-7 cells were all significantly lower than those in si-NC groups (P<0.01, P<0.05, Figure 3A). The western blot assay showed that the p21 protein levels in si-WSTF MDA-MB-231 and MCF-7 cells were all significantly reduced compared to those in si-NC groups（P<0.01, P<0.01, Figure 3B). The mRNA and protein levels of CCL2 in MDA-MB-231 and MCF-7 cells were also decreased by si-WSTF (mRNA: P<0.05, P<0.05, Figure 3C; protein: P<0.05, P<0.01, Figure 3D). These results indicated that WSTF positively regulated p21 and CCL2 expressions in BC cells.

Figure 3: Effects of WSTF knockdown on the expressions of p21 and CCL2 in MDA-MB-231 cells and MCF-7 cells



Note: MDA-MB-231 and MCF-7 cells were transfected with si-WSTF and si-NC, respectively. The mRNA levels of p21(A) and CCL2(C) in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. The protein levels of p21(B) and CCL2(D) in the indicated groups were detected by western blotting, using GAPDH as an internal reference. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, * P<0.05, ** P<0.01

Overexpression of P21 Reversed the Inhibition Effect of Si-WSTF on Migration Ability of BC Cells

Furthermore, we analyzed whether WSTF affected the migration abilities of BC cells by regulating expressions of p21 and CCL2.

MDA-MB-231 and MCF-7 cells were transfected with N1 plasmid containing open reading frame (ORF) of p21 (p21) or N1 plasmid containing ORF of CCL2 (CCL2) and empty N1 plasmids (N1), respectively.

It was confirmed that p21 was upregulated in p21 MDA-MB-231 and p21 MCF-7 cells at 24h post-transfection (P<0.01, P<0.01, Figure 4A).

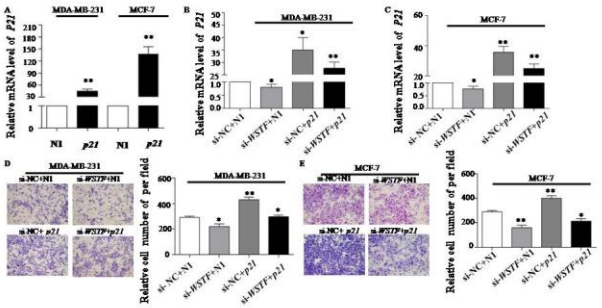
MDA-MB-231 and MCF-7 cells were transfected with siRNA control+empty plasmid control (si-NC+N1), WSTF siRNA+empty plasmid (si-WSTF+N1), siRNA control+p21 N1 plasmid (si-NC+p21), WSTF siRNA+p21 N1 plasmid (si-WSTF+p21), respectively. The RT-qPCR results showed that the transfection of p21 plasmid reversed the decreasing effect of WSTF knockdown on the p21 expression in MDA-MB-231 cells (si-WSTF+N1 vs. si-NC+N1, P<0.05; si-NC+p21 vs. si-NC+N1, *P*<0.05; si-WSTF+p21 vs. si-WSTF+N1, *P*<0.01; Figure 4B).

As well as in MCF-7 cells (si-WSTF+N1 vs. si-NC+N1, *P*<0.05; si-NC+p21 vs. si-NC+N1, *P*<0.01; si-WSTF+p21 vs. si-WSTF+N1, *P*<0.01; Figure 4C).

Transwell assay showed that overexpression of p21 reversed the si-WSTF inhibition effect on migration ability of BC MDA-MB-231 cells (si-WSTF+N1 vs. si-NC+N1, P<0.05; si-NC+p21 vs. si-NC+N1, P<0.01; si-WSTF+p21 vs. si-WSTF+N1, P<0.05; Figure 4D),

as well as MCF-7 cells (si-WSTF+N1 vs. si-NC+N1, P<0.01; si-NC+p21 vs. si-NC+N1, P<0.01; si-WSTF+p21 vs. si-WSTF+N1, P<0.05; Figure 4E).

Figure 4: Effects of WSTF knockdown or p21 overexpression on migration abilities in MDA-MB-231 and MCF-7 cells



Note: (A): MDA-MB-231 and MCF-7 cells were transfected N1 plasmid containing open reading frame (ORF) of p21 (p21) and p21 negative control (N1), respectively. The mRNA levels of p21 in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. MDA-MB-231 and MCF-7 cells were transfected with siRNA control + plasmid control (si-NC + N1), WSTF siRNA + plasmid control (si-WSTF + N1), siRNA control + p21 plasmid (si-NC + p21), WSTF siRNA + p21 plasmid (si-WSTF + p21). (B-C): The mRNA levels of p21 in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. (D): Migration ability of each group cells of MDA-MB-231 was detected by transwell assay. (E): Migration ability of each group cells of MCF-7 was detected by transwell assay. The above experiments are three independent repeated experiments, and the results are expressed as mean ± SD, * *P*<0.05, ** *P*<0.01.

Overexpression of CCL2 Reversed the Inhibition Effect of Si-WSTF on Migration Ability of BC Cells

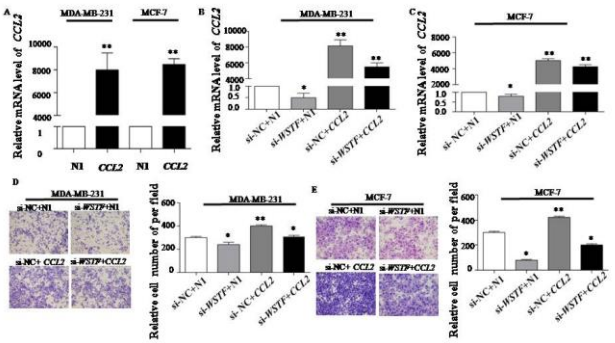
It was also confirmed that CCL2 was upregulated in CCL2 MDA-MB-231 and CCL2 MCF-7 cells at 24h post-transfection (P<0.01, P<0.01, Figure 5A).

As shown in Figure 5B, transfection of CCL2 plasmid reversed the decreasing effect of WSTF knockdown on the CCL2 expression in MDA-MB-231 cells (si-WSTF+N1 vs. si-NC+N1, P<0.05; si-NC+CCL2 vs. si-NC+N1, P<0.01; si-WSTF+CCL2 vs. si-WSTF+N1, P<0.01; Figure 5B), as well as in MCF-7 cells (si-WSTF+N1

vs. si-NC+N1, P<0.05; si-NC+CCL2 vs. si-NC+N1, P<0.01; si-WSTF + CCL2 vs. si-WSTF + N1, P<0.01; Figure 5C).

Moreover, transwell assay showed that CCL2 overexpression reversed the si-WSTF inhibition of migration ability of MDA-MB-231 cells (si-WSTF+N1 vs. si-NC+N1, P<0.05; si-NC+CCL2 vs. si-NC+N1, P<0.01; si-WSTF+CCL2 vs. si-WSTF+N1, P<0.05; Figure 5D), as well as MCF-7 cells (si-WSTF+N1 vs. si-NC+N1, P<0.01; si-NC+CCL2 vs. si-NC+N1, P<0.01; si-WSTF+CCL2 vs. si-NC+N1, P<0.01; Figure 5E). These data illustrated that hyper-expressed WSTF in BC promoted the cell migration ability by upregulating p21 and CCL2 expressions.

Figure 5: Effects of WSTF knockdown or CCL2 overexpression on migration abilities in MDA-MB-231 and MCF-7 cells



Note: (A): MDA-MB-231 and MCF-7 cells were transfected N1 plasmid containing ORF of CCL2 (CCL2) and CCL2 negative control (N1), respectively. The mRNA levels of CCL2 in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. MDA-MB-231 and MCF-7 cells were transfected si-NC + N1, si-WSTF + N1, siRNA control + CCL2 plasmid (si-NC + CCL2), WSTF siRNA + CCL2 plasmid (si-WSTF + CCL2). (B-C): The mRNA levels of CCL2 in the indicated groups were detected by RT-qPCR, using GAPDH as an internal reference. (D): Migration ability of each group cells of MDA-MB-231 was detected by transwell assay. (E): Migration ability of each group cells of MCF-7 was detected by transwell assay. The above experiments are three independents repeated experiments, and the results are expressed as mean ± SD, * P<0.05, ** P<0.01.

CONCLUSIONS

In this study, we found that WSTF was highly expressed in human BC MDA-MB-231 and MCF-7 cell lines. Knockdown of WSTF inhibited the migration of MDA-MB-231 and MCF-7 cells, as well as the expressions of p21 and CCL2.

Overexpression of p21 or CCL2 could partially reverse the inhibition of WSTF knockdown on BC cell migration ability.

Previous studies had shown that overexpression of WSTF promoted the proliferation, migration and invasion of lung cancer cells.[5] Other studies had shown that WSTF promoted proliferation and invasion of cervical cancer,[13] and glioblastoma cells,[14] and promoted the progress of gastric cancer.[15] Therefore, WSTF plays an oncogene in the above cancers.

Studies had shown that the p21 gene was highly expressed in BC and ovarian clear-cell carcinoma, and its expression was correlated with malignant behaviors. [16,17] On the other hand, the loss of p21 expression had been reported in several solid tumors and associated with metastasis and poor prognosis of different cancers, including gastric, bladder, non-small cell lung and prostate cancers.[18-21] Together with our data, the function of p21 in regulating the migration ability of cancer cells should be further studied.

Several studies had reported that CCL2 promoted the malignant behavior of different tumors including BC.[22-24] This study showed that CCL2 was a downstream gene of WSTF.

It was reported that CCL2 was regulated by transcript factor signal transducer and activator of transcription 3 (STAT3) and involved in regulating immunity of cancer-associated fibroblasts.[25] Wherefore, CCL2 played a regulatory role by signaling pathways.

To sum up, the present study demonstrated that WSTF was upregulated in BC cells and tissues, WSTF knockdown suppressed migration abilities of BC cells, which could be reversed by overexpression of p21 and CCL2. This study provides a new epigenetic basis for the study of BC cells migration mechanism

DECLARATIONS

Funding

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Conflict of Interest

None declared.

Authors' Contributions

LYF and ZJH conceived and designed the study. JJX prepares experimental materials. WZ, LD and WSQ analyzed the data. WZ wrote the manuscript and carefully considered the research plans and contents. LBF, WYQ, WY and ZLN confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Consent for Publication

Not applicable.

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