Long non-coding RNA UCA1 promotes papillary thyroid cancer cell proliferation via miR-204-mediated BRD4 activation

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Abstract. Long non-coding RNA (IncRNA) urothelial carcinoma-associated 1 (UCA1) has been used in tumor development and progression in many types of cancer. However, the function and mechanism underlying the action of UCA1 in papillary thyroid cancer (PTC) remains unclear. Therefore, these topics were investigated in the present study by in vitro and in vivo experiments. It was demonstrated that the expression level of UCA1 was more significantly upregulated in PTC cell lines and tissues when compared with the immortal human thyroid follicular cell line and adjacent normal tissues, respectively. UCA1 knockdown significantly inhibited PTC cell viability, colony formation and the bromodomain containing 4 (BRD4) expression level in vitro, and retarded PTC tumor growth in vivo. In the previous study, microRNA (miR)-204 inhibited thyroid cancer progression and was regulated by UCA1 in other types of cancer. In addition, by conducting dual luciferase reporter assays, it was confirmed that miR-204 directly binds to UCA1 and the 3'-untranslated region of BRD4. Furthermore, UCA1 competed with BRD4 for miR-204 binding, miR-204 knockdown enhanced BRD4 expression, which can be partially restored by short hairpin-UCA1. The results of the present study illustrated that UCA1 promotes PTC progression by acting as a competing endogenous RNA by sponging miR-204. In conclusion, UCA1 may be regarded as an oncogenic IncRNA, promoting PTC cell proliferation, and be a potential target for human PTC treatment.

Introduction

Thyroid cancer is the most common tumour of the endocrine system, and its incidence rate has dramatically increased over the past several decades (1). Thyroid cancer incidence is rapidly increasing in the USA; in 2017, its estimated annual diagnosis rate and annual mortality rate were 56,870 people and 2,010 cases, respectively (2). Papillary thyroid carcinoma (PTC) accounts for the majority of thyroid cancers and generally exhibits favourable prognosis (3). However, the recurrence rate is approximately 15% in 3 years (4). Recurrent PTC has limited therapeutic options and poor prognosis, and this disease is currently the leading cause of death in patients with PTC. Therefore, our understanding of the mechanisms underlying the pathogenesis of PTC should be improved, and further effective therapeutic approaches should be developed.

Long non-coding RNAs (lncRNAs) are a diverse class of RNAs with a length of more than 200 nucleotides that do not encode proteins (5). Genome-wide transcriptional analysis has revealed the high prevalence of IncRNAs in all transcripts (5). IncRNAs are important epigenetic modulators with sensory, guiding and scaffolding capacities (6). Thus, lncRNAs have gained increasing attention as a critical regulator of normal physiology and disease development, particularly in proliferation and migration (7). IncRNA expression is dysregulated in many diseases affecting different tissue types (7). lncRNAs can be detected in various human body fluids, such as serum and plasma, suggesting that IncRNAs may serve as new biomarkers for disease diagnosis and prognosis without invasive procedures (8).

Urothelial carcinoma-associated 1 (UCA1) is a IncRNA that shows potential as a biomarker for cancer. The UCA1 gene is located at chromosome 19p-13.12 and dysregulated in breast cancer, oesophageal cancer, gastric cancer and bladder cancer (9-13). UCA1 depletion can attenuate the migratory ability of various cancers (9-13), whereas UCA1 overexpression can enhance ovarian cancer cell migration and invasion (14), suggesting that UCA1 functions as a pivotal regulator of cellular migration and invasion. Nevertheless, up to now, there is no relevant report about the relationship between UCA1 and the progression of PTC. Therefore, the role and underlying mechanism of UCA1 in PTC should be elucidated.

Previous studies have showed that miR-204 plays a protective role by inhibiting thyroid cancer cell proliferation and may identify new targets for anti-cancer treatment (15,16). Moreover, in the recent studies has showed that UCA1 can...
directly regulated the expression of miR-204 in prostate cancer, colorectal cancer and oesophageal cancer (9-11,17). However, the correlation of UCA1 and miR-204 remains elusive in PTC. Therefore, there is a vital necessity to illustrate the relationship of UCA1 and miR-204 in PTC.

In this study, we reported an interaction between UCA1 and miR-204, which regulates PTC cell growth by targeting BRD4. Our study demonstrated that the UCA1/miR-204/BRD4 signalling pathway might be a novel therapeutic target for patients with PTC.

Materials and methods

Cell culture and tissue collections. The human PTC cell lines (TPC-1 and BCPAP) and immortal human thyroid follicular cell line (Nthy-ori3-1) were obtained from the Cell Bank of China (Shanghai, China). These cell lines were authenticated via short-tandem repeat profiling performed by BMR Genomics. BCPAP usually known as a PTC cell line is derived from a poorly differentiated PTC, however, recent study has showed that the mRNA expression profile of BCPAP were closer to anaplastic thyroid cancer cells (18). In this study, BCPAP serves as poorly differentiated papillary thyroid cancer cell line to further confirm the experiments results of TPC-1 and not affect the outcomes of this study. The cells were cultured in DMEM (HyClone; GE Healthcare Life Sciences, Shanghai, China) supplemented with 10% foetal bovine serum (HyClone; GE Healthcare Life Sciences) in a humidified atmosphere with 5% CO₂ and humidified atmosphere of 95% at 37°C. Human PTC specimens and their adjacent normal thyroid tissues were obtained from each patient, and the study protocol and consent procedures were approved by the Ethics Committee of the Jining Medical University (Shandong, China).

Cell transfection. LV3 (H1/GFP&Puro) vector was synthesized for Lv-shRNAUCA1 (Guangzhou Ribobio Co., Ltd., Guangzhou, China). A nontarget scrambled oligonucleotide served as the negative control. The shRNA sequences used in the present study were as follows: shUCA1, 5'-GCCACCUACAUAAAGCGU AdTdT-3' and shcontrol, 5'-CAGUACUUUUGUGUAGUACAA -3'. miR-204 mimic (miR10002265-1-5), miR-NC (miR01201-1-5), inhibitor (miR20022693 -1-5) and anti-NC (miR02201-1-5) were obtained from Guangzhou Ribobio Co., Ltd.. Transfection was performed by using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. First-strand cDNA was generated by reverse transcribing the total cellular RNA with M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The SYBR Premix Ex Taq™ kit (Takara, Bejing, China) at 94˚C for 30 sec, followed by 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. Relative mRNA expression was analyzed as the inverse log of ΔΔCq and was normalized to the reference gene (19).

UCA1: Forward 5’-TTTGGCAGCGCTAGGTTTAAT-3’, Reverse 5’-TTGTCCTCATTTCCATCAT-3’; miR-204 Forward 5’-GTCCCTGTGTCATCCT-3’, Reverse 5’-CAGTGCAGGGTCCGAGGTA-3’, U6 Forward 5’-GTCCCTGTGTCATCCT-3’, Reverse 5’-CAGTGCAGGGTCCGAGGTA-3’; BRD4 Forward 5’-CATGGACAGGGTCCGAGGTA-3’, Reverse 5’-CATGGACAGGGTCCGAGGTA-3’, GAPDH Forward 5’-TGTCAGACCTGCCGAGGTGTT-3’, Reverse 5’-GTGGTGAGATATTAGAGGG-3’.

Cell viability assay. Cell viability was examined using CCK-8 assay. Briefly, TPC-1 and BCPAP cells transfected with either shUCA1 or shcontrol were plated onto 96-well plates with 3,000 cells/well. After culturing at an indicated time (0, 24, 48 and 72 h), 10 µl of CCK-8 solution was added into each well at 37°C. After 3 h, absorbance was determined using a microplate spectrophotometer at a wavelength of 450 nm.

Colony formation assay. The TPC-1 and BCPAP cells transfected with either shUCA1 or shcontrol (500 cells/well) were plated into 6-well plates. After 1 week, the colonies were fixed with methanol and stained with 0.1% crystal violet for 20 min, and the images of the stained colonies were captured by using a CKX41 light microscope (Thermo LabSystems, Vantaa, Finland). The number of the colonies was counted by using the images.

Migration and invasion assay. Migration and invasion ability was examined by using Transwell assay. For the invasion assay, the upper sides of the filters were coated with 50 µl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Indicated cells were plated at a density of 5x10⁴ per well in the upper chamber without serum. Cells were incubated for 12 h for the migration assay and 24 h for the invasion assay. After

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from cells and tissues was isolated with a TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. First-strand cDNA was generated by reverse transcribing the total cellular RNA with M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The SYBR Premix Ex Taq™ kit (Takara, Bejing, China) at 94˚C for 30 sec, followed by 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. Relative mRNA expression was analyzed as the inverse log of ΔΔCq and was normalized to the reference gene (19).
the cells were incubated at the exact time at 37°C, the inserts were washed with PBS, and cells on the upper surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with 4% formaldehyde for 20 min, stained with 0.1% crystal violet solution and imaged using a CKX41 light microscope.

**Dual luciferase reporter assay.** TPC-1 and BCPAP cells cultured in 24-well plates were cotransfected with luciferase reporter plasmids (wt-BRD4, mut-BRD4 containing miR-204 binding site, wt-UCA1 and mut-UCA1 containing miR-204 binding site) and miRNA mimics or inhibitor. After 48 h, luciferase activity was measured via dual-luciferase reporter assay system according to the manufacturer’s instruction (Promega Corporation).

**In vivo assay.** All the procedures were carried out in accordance to the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Care and Use Committee of the Jining Medical University (Jining, China). Five-week-old female BALB/c nude mice were purchased from the Yangzhou Laboratory Animal Centre and maintained in a SPF environment. Subcutaneous tumour xenografting was performed by subcutaneously injecting mice with 100 µl of PBS containing 5x10⁵ BCPAP cells that had been transfected with either shUCA1 or shcontrol. Tumour volume (mm³) was calculated every 7 days by using the following equation: V = 0.5 x length x width². After 42 days, the mice were euthanized, and the tumors were isolated, weighed, photographed and then processed for further study.

**Immunohistochemistry.** Paraffin-embedded sections of tumor tissue (4 µm thick) were deparaffinized in xylene, rehydrated via graded alcohol solutions, blocked in methanol containing 3% hydrogen peroxide for 10 min at room temperature, and then incubated with mouse BRD4 antibody (ab84776, 1:500; Abcam, Cambridge, MA, USA) at 4°C overnight. Following rinsing with PBS solution, biotinylated goat anti-rabbit serum IgG (ab6785, 1:2,000; Abcam, Cambridge, MA, USA) was used as secondary antibodies and streptavidin peroxidase complex reagent were applied for 1 h at room temperature. Finally, the sections were incubated in a 3,3′-diaminobenzidine solution at room temperature for 10 min and then counterstained with hematoxylin for 3 min at room temperature. Ten randomly selected visual fields per section were examined under a light microscope to evaluate the BRD4 expression.

**Statistical analysis.** All experiments were performed three times. The data were presented as the mean ± standard deviation. Differences between two groups were assessed using two-tailed Student’s t-test. Data from >2 groups were analyzed using one way analysis of variance with the post hoc Tukey’s test. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Differences were considered statistically significant when P<0.05.

**Results**

**UCA1 is upregulated in PTC tissues and cell lines.** To evaluate the role of UCA1 in PTC, we first detected the expression level of UCA1 in 30 pairs of human PTC specimens and corresponding adjacent non-tumor tissues were determined by using qPCR. Results illustrated that the expression of UCA1 was significantly higher in tumor tissues (Fig. 1A). Furthermore, the expression level of UCA1 in PTC cell lines (TPC-1 and BCPAP) was significantly enhanced compared to that in the human immortal follicular thyroid cell Nthy-ori3-1 (Fig. 1B). Therefore, UCA1 might play a critical role in the progression of PTC.

**UCA1 silencing decreased cell viability, proliferation in PTC cell lines.** UCA1 knockdown was achieved by transfection with shUCA1 in BCPAP and TPC-1, as verified via qPCR assays (Fig. 2A and B). The CCK-8 results showed that the cell viability of BCPAP and TPC-1 cells was significantly decreased after UCA1 knockdown (Fig. 2C and D). In addition, the colony formation assay results illustrated that the proliferation of UCA1 silencing cells was remarkably decreased (Fig. 2E and F). The Transwell assay illustrated that the migration and invasion of UCA1 silencing cells was significantly decreased (Fig. 2G and H).

Previous study has showed that BRD4 is involved in the progression of PTC (20). BRD4 silencing decreased the tumor viability, proliferation in vitro and in vivo (20). Here we also monitored the protein levels of BRD4 in BCPAP and TPC-1 cells, in response to UCA1 silencing. The western blot
and qPCR results showed that UCA1 silencing significantly decreased the expression level of BRD4 (Fig. 3A-D). These results illustrated that UCA1 might enhance PTC progression via the activation of BRD4.

miR-204 could negatively regulate BRD4 expression. To discover the underlying mechanism by which UCA1 regulated PTC progression, TargetScan (http://www.targetscan.org/vert_71/) was used. BRD4 was predicted to be a target of miR-204. miR-204 has been demonstrated to serve as a tumor suppressor gene in some kinds of cancer including thyroid cancer (15,16). Moreover, recent studies have showed that UCA1 can directly regulate the expression of miR-204 in some types of cancer (9,11,17). BCPAP and TPC-1 cells were transfected with miR-204 mimic, miR-NC, miR-204 inhibitor or NC-inhibitor respectively. The expression level of miR-204 was examined via qPCR (Fig. 4A and B). The results showed that the mRNA expression of BRD4 was significantly decreased by miR-204 overexpression while enhanced by miR-204 inhibition (Fig. 4C and D). The protein expression levels of BRD4 in BCPAP and TPC-1 cell lines in response to miR-204 change were then examined by Western blot. Results showed that the protein expression of BRD4 was decreased by miR-204 mimic while increased by miR-204 inhibitor (Fig. 4E and F). These data showed that miR-204 could negatively regulate BRD4 expression in PTC cell lines.

UCA1 competed with BRD4 for miR-204 binding. We demonstrated that miR-204 negatively regulated BRD4 expression; then we illustrated the correlation between UCA1 and miR-204.

Figure 2. UCA1 promotes PTC cell proliferation. (A) BCPAP and (B) TPC-1 cells were transfected with shUCA1 and shcontrol, and the expression of UCA1 was detected by reverse transcription-quantitative polymerase chain reaction. (C-F) Cell viability and proliferation was separately assessed by (C and D) Cell Counting Kit-8 and (E and F) colony formation assays. (G and H) The migration and invasion of (G) BCPAP and (H) TPC-1 cells was examined via Transwell assay (magnification, x100). *P<0.05 vs. shcontrol/as indicated. UCA1, urothelial carcinoma-associated 1; PTC, papillary thyroid cancer; sh-, short hairpin RNA.
Figure 3. UCA1 regulates BRD4 expression via miR-204. BCPAP and TPC-1 cells were co-transfected with shUCA1 and miR-204 inhibitor (anti-miR-204); the (A and B) mRNA and (C and D) protein levels of BRD4 were then examined via reverse transcription-quantitative polymerase chain reaction and western blot assays, respectively. *P<0.05, as indicated. UCA1, urothelial carcinoma-associated 1; sh-, short hairpin RNA; BRD4, bromodomain containing 4; miR, microRNA.

Figure 4. miR-204 negatively regulates BRD4 expression. (A and B) Relative expression levels of miR-204 were determined in thyroid cancer cells transfected with miR-204 mimic, miR-NC, miR-204 inhibitor or NC-inhibitor respectively by RT-qPCR. (C and D) The mRNA expression levels of BRD4 in (C) BCPAP and (D) TPC-1 cells in response to miR-204 expression alteration were also determined by RT-qPCR. (E and F) The protein level of BRD4 in (E) BCPAP and (F) TPC-1 cells in response to miR-204 expression alteration were determined via western blot assays. *P<0.05, as indicated. UCA1, urothelial carcinoma-associated 1; sh-, short hairpin RNA; BRD4, bromodomain containing 4; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
in PTC. The expression level of UCA1 in response to miR-204 inhibition and overexpression in BCPAP and TPC-1 cell was examined via qPCR. Results showed that UCA1 was inhibited by miR-204 overexpression while enhanced by miR-204 inhibition (Fig. 5A and B). In addition, qPCR data illustrated that the expression of miR-204 was remarkably enhanced while UCA1 expression was downregulated (Fig. 5C and D). These results demonstrated a dual regulation between UCA1 and miR-204. According to online tools, UCA1 shared a same binding site in miR-204 with BRD4. Then luciferase assays were performed to discover the correlations between miR-204 and UCA1. Luciferase reporter gene vectors (wt-BRD4 3'UTR and mut-BRD4 3'UTR containing a 6 bp mutation in the predicted binding sites of miR-204) were constructed. (F and G) Following overnight culture, cells were co-transfected with the indicated vectors and miR-204 mimics or miR-204 inhibitor, respectively. Luciferase assays were performed 48 h following transfection to determine the luciferase activity. *P<0.05, as indicated. UCA1, urothelial carcinoma-associated 1; sh-, short hairpin RNA; BRD4, bromodomain containing 4; miR, microRNA; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WT, wild type; MUT, mutant; UTR, untranslated region.

UCA1 regulated BRD4 expression via miR-204. To validate whether UCA1 regulated BRD4 expression through miR-204, we cotransfected BCPAP and TPC-1 cells with miR-204 inhibitor and shUCA1, and examined the expression of BRD4 by western blot and qPCR. The results showed that the expression of protein and mRNA of BRD4 were increased by miR-204 inhibition, decreased by shUCA1 (Fig. 3A-D). The promotive effect of miR-204 inhibition on the expression of BRD4 could be partially abolished by shUCA1 (Fig. 3A-D). These results demonstrated that UCA1 could regulate BRD4 expression through miR-204.

Knockdown of UCA1 inhibits tumor growth in vivo. To further illustrate the effects of UCA1 on the PTC growth in vivo, we applied a xenograft model in which the BCPAP cells transfected with shUCA1 or shNC were subcutaneously
injected into the flanks of athymic mice and measured the tumor in a period time. The results showed that tumors formed by transfected shUCA1 grew more slowly than those formed by transfected shNC (Fig. 6A-C). Furthermore, qPCR results showed that the BRD4 expression levels in the shUCA1 tumour xenografts were lower than those in the control xenografts (Fig. 6D). In addition, the qPCR results showed that the expression level of miR-204 was significantly downregulated in shUCA1 transfected tumor compared to shNC transfected tumor (Fig. 6E). At last, histologic analysis showed that shUCA1 tumors had significantly fewer BRD4 positive cells than the shNC tumors (Fig. 6F and G). The in vivo results also demonstrated that UCA1 might regulate BRD4 via miR-204.

**Discussion**

In the present study, we confirmed the oncogenic role of UCA1 in the progression of PTC. Initially, we demonstrated that lncRNA UCA1 was significantly increased in PTC cell lines and tissues. Then, we showed that UCA1 silencing inhibited the viability, proliferation, migration and invasion of PTC cells in vitro and in vivo. Finally, we observed that UCA1 could sponge miR-204 and modulate BRD4 expression.

BRD4 is an epigenome reader and member of the bromo-domain and extra-terminal family of proteins, which consist of two bromodomains in tandem and an extra-terminal domain. BRD4 promotes cell cycle progression, regulates cell growth and transcription and plays a critical role in the tumour progression of various cancers via different molecular mechanisms (21-28). BRD4 inhibition with JQ1 significantly prevents PTC progression in vitro and in vivo (20). The BRD4 expression in breast cancer is regulated by miR-599 (29). However, how BRD4 is aberrantly dysregulated in PTC is unclear, and the relation between miRNA and BRD4 has yet to be established. miR-204 plays a protective role by inhibiting thyroid cancer cell proliferation and may identify new targets for anti-cancer treatment (15). In the present study, luciferase assay and Western blot analysis confirmed that BRD4 was a direct target gene of miR-204.
IncRNAs are transcribed RNA molecules with a length of more than 200 nucleotides that lack a substantial protein-coding potential. They can regulate protein-coding genes at epigenetic, transcriptional and post-transcriptional levels and participate in physiological processes. Various IncRNAs are frequently aberrantly expressed in cancers, and their differential expression is closely related to tumor progression; therefore, they serve as oncogenes or tumor suppressor genes (28). Further studies should be conducted to clarify the biological function and molecular mechanisms of IncRNAs in tumor progression.

UCA1 is an identified IncRNA that promotes tumor progression in a wide range of tumor types, including bladder cancer, breast cancer, hepatocellular carcinoma, colorectal cancer and gastric cancer (9,30-32). However, the role of UCA1 in PTC has yet to be illustrated. Initially, we investigated the mRNA expression of UCA1 in PTC tissues and cell lines and found that UCA1 expression was significantly higher in PTC tissues and cell lines than in normal tissues. Moreover, UCA1 levels in PTC tissues were positively correlated with advanced clinical stages and lymph node metastasis, but not with age, gender, or tumor size (data not shown). These data suggested that UCA1 functioned as an oncogene in PTC, and this finding is consistent with previous results on other tumors (9,30-32). To investigate the molecular mechanism by which UCA1 affects PTC proliferation and migration, we monitored the protein level of BRD4 when the UCA1 expression was downregulated by using shUCA1 in thyroid cancer. We showed that UCA1 silencing reduced the BRD4 protein expression, indicating the involvement of BRD4 in the regulation of UCA1 cell proliferation and invasion. A previous study demonstrated that IncRNAs act as competing endogenous RNAs to mediate miRNAs, and IncRNA-miRNA interaction regulates tumor progression through the regulation of oncogenes or tumor suppressor genes (33). UCA1 is related to miR-204 via the regulation of different downstream target genes (9-11). In the present study, we revealed the interaction between UCA1 and miR-204 in PTC for the first time; that is, UCA1 and miR-204 could negatively regulate each other. In this reciprocal negative regulation loop, the overexpression of UCA1 represses the expression of miR-204 to upregulate the expression of BRD4, thereby enhancing thyroid cancer cell growth and migration and invasion. On the other hand, the miR-204 represses the expression of UCA1 and decrease the expression of BRD4, so that to inhibit the tumor growth and metastasis. The equilibrium might depend on the tumour microenvironment and the type of cancer. We also showed that miR-204 could directly control the BRD4 expression. Given this result, we investigated whether miR-204 could regulate UCA1 and BRD4 through direct targeting. Dual luciferase assays revealed that UCA1 could bind to miR-204. miR-204 could also bind to the 3’UTR of BRD4, suggesting that UCA1 might compete with BRD4 for miR-204 binding to inhibit miR-204, to promote BRD4 expression and to affect PTC progression. To further discover whether BRD4 could regulate the expression of UCA1 and miR-204, we used siBRD4 to knockdown the expression of BRD4, and did not discover that BRD4 could regulate the expression of miR-204 and UCA1 in PTC cell lines (data not shown). These data demonstrated that BRD4 was a downstream target gene of UCA1/miR-204 axis, and illustrated that drug resistance to BRD4 might select the upstream gene UCA1 or miR-204 as alternative therapy target.

In conclusion, the UCA1/miR-204/BRD4 axis plays a critical role in PTC cell proliferation and invasion and shows potential for therapeutic applications in patients with thyroid cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

DL and DH conceived and designed the experiments. CC, JC, ZH and YW conducted all of the experiments. YW wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jining Medical University (Jining, China). All patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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