The Expression of Ptch1 and Ptch2 in the Stenotic Tissue of Congenital Ureteropelvic Junction Obstruction in Children

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Abstract

Objective

Ptch1 and Ptch2 are expressed in tubular epithelium and stromal cells adjacent to the UPJ. They mediated inhibition of smoothened, a transmembrane protein expressed on the cell surface. If the pathway was disturbed, UPJO might be onset. The aim of this study was to determine the expression of Ptch1 (P1) and Ptch2 (P2) in stenotic segments in children with congenital ureteropelvic junction obstruction (UPJO) versus in normal control subjects.

Materials and methods

Stenotic segments of ureter tissues were obtained from 20 UPJO patients. Extrinsic stenosis, such as vessel and ureteral polyp, were excluded. The control ureter specimens were obtained from 10 patients with Wilm's tumor, and the tissues were confirmed histologically to be unaffected. Immunofluorescence, Western blot and real-time PCR were used to investigate the expression of P1 and P2. Statistical methods were used to find the differences between the two groups

Results

P1 and P2 were identified that located in the cytoplasm of smooth muscle in two groups through Immunohistochemistry. However, there were no statistical differences between the two groups in P1 and P2 with immunohistochemistry (P=0.31 and P=0.3, respectively). Meanwhile there were no statistical differences with Western blot (P=0.75 and P=0.9, respectively) and real-time PCR (P=0.52 and P=0.45, respectively). But we found that in the immunofluorescence P1 were diffused in controls and mainly surrounding the nucleuses of smooth muscle cells in UPJO.

Conclusions

The expression of P1 and P2 between the two group had no statistically significant. P1 mainly surrounding the nucleuses of smooth muscle cells in UPJO. The P1 pathway might be disturbed by the abnormal distribution of P1 rather than the quantity.

Background

Ureteropelvic junction obstruction (UPJO) is the most common form of congenital urinary tract obstruction (1). Most cases of UPJO are caused by intrinsic narrowing of the ureter at the pelvic-kidney junction, while the underlying pathogenic mechanisms are undefined exactly (2). Researchers found that Sonic hedgehog (Shh) signaling pathway was involved in kidney and ureter development during embryonic period (3,4). Several researchers had found that the pathway was disturbed by experimental models and UPJO children (5).

Shh pathway was important in renal development. There were Shh and its receptor Patched in the tubular epithelium and stromal cells of the UPJ, (6). Shh bound to Ptch and relieved Smoothened, which was a transmembrane Ptch-mediated inhibition protein expressed on the cell surface (5). Full-length Gli3 proteins translocated to the nucleus after bounding, acting as transcriptional activators (7). If the pathway was disturbed, UPJO might be onset.

There are two Ptch homologs in mammals, Ptch1 (P1) and Ptch2 (P2), both of which bind to Shh ligands with similar affinity (8). Despite rigorous investigations, the Ptch in UPJO children remains unclear, and whether there was variation yet to be proved (9). The expression of P1 and P2 were compared in hydronephrotic model or contiguous segments in same ureters of UPJO patients, but the actual human ureteropelvic junction segments were not (5,10).

Here, we investigated the expression of P1 and P2 in stenotic segments in children with PUJO versus in normal control subjects using immunohistochemistry, Western blot and real-time PCR methods, aiming to find the possible pathogenic mechanisms in congenital hydronephrosis due to UPJO. The control specimens were obtained from ureteropelvic junction segments of Wilm's tumor patients.

Methods

Patients and control samples

This study was approved by the Ethics Committee (2019-k-368). Stenotic segments of ureter tissues were obtained from 20 UPJO patients. The diagnosis was based on the accessory examination. Surgical indications followed EUA guideline. Intrinsic stenosis was confirmed during operation. Extrinsic stenosis, such as vessel and ureteral polyp, were excluded. The control ureter specimens were obtained from 10 patients with Wilm's tumor, and the tissues were confirmed histologically to be unaffected. The samples of ureteropelvic junction were immediately stored at -80℃ immediately after operation.

Immunofluorescence Of P1 And P2

Immunofluorescence was performed on 4 μm paraffin sections; Dako serum-free protein block was used for blocking, primary and secondary incubation buffers. Whole-mount immunofluorescence was performed as usual. Antibodies were used: P1 (Primary antibodies: goat, lot: ab39266; Fluorescent secondary antibodies: CY3 Labeled rabbit anti-goat IgG) and P2 (Primary antibodies: rabbit, lot: ab238338; Fluorescent secondary antibodies: 488 Labeled goat anti- rabbit IgG) (Abcam company, 1:100). Every patient's tissue was divided into three parts, every part was photographed under fluorescent microscopy in five different areas and calculated the average value.

Western Blot Of Ptch1 And Ptch2

Pre-cooled RIPA protein extraction reagent (50 mMTris-HCl(pH 7.4), 150 mM NaCl, 1% np-40, 0.1% SDS) were added to Protease inhibitor cocktail (Roche) (phosphorylated protein needs to be added to phosphatase inhibitor at the same time) (according to the volume ratio of 50:1).The tissue was cut into pieces, and 50 mg of tissue was added into 500 ul lysate. The tissue was centrifuged at 13000 rpm for 20 min. Remove the supernatant and store at -80 degrees for Wb. The separation gel was prepared according to the molecular weight of the target protein. Then electrophoresis, incubation of primary and secondary antibodies and film exposure were performed. Primary antibodies were P1 (goat, 1:300) and P2 (rabbit, 1:1000), secondary antibodies were goat anti rabbit IgG and goat anti mous IgG (1:10000).

RNA Isolation And RT-PCR

ABI 7500 fluorescence quantitative PCR instrument was used, and 2-train ct method was used for relative quantitative analysis of the data. Quantitative real-time PCR was accomplished with SYBR Premix Ex Taq (TaKaRa) on LightCycler-GmbH D-68298 (Roche Molecular Biochemicals) under the following conditions: 95℃ for 10 s, 45 cycles of 95℃ for 5 s, 58℃ for 20 s; 65℃ for 15 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity.Primer sequences were as follows: P1 forward, 5’-CGCTCTGGAGCAGATTTCCA-3’, reverse, 5’-CTCGTCCTCCAACTTCCACC3; P2 forward 5’- GCCGCCAGAGGTGATACAG-3’, reverse, 5’- GTGTGTCTGATGAGGGGGTG-3’. Beta actin 5’-ACAGAGCCTCGCCTTTGCC-3’, reverse, 5’-GATATCATCATCCATGGTGAGCTGG-3’

Statistical analysis

All data were presented as mean ± SD. Significance of differences was evaluated by using two-sample t test, Linear regression analysis was used to test the correlation among the parameters, P value < 0.05 was considered to be statistically significant.

Results

The UPJO patients ranged from 6 month to 12.5 years old, with a mean age of 3.3 years. The control group ranged from 1.2 month to 7 years old, with a mean age of 3.2 years. No statistically differences were found between the two groups with regard the age of patients (P = 0.28).

Immunofluorescence

P1 was specifically stained red and P2 was green, the nucleuses of smooth muscle cells were stained blue. In UPJO group, expression of P1 and P2 were found in stenotic segments (Fig. 1A and 1C). In control group, expression of P1 and P2 were detected in ureteropelvic junction segments (Fig. 1B and 1D), linear regression analysis between P1 and P2 with age were P = 0.63 and P = 0.33, respectively. There were no statistical differences between the two groups in P1 and P2. Results of the immunofluorescence and P values between the two groups were summarized in Table 1.

Table 1
Results and P values of the immunofluorescence, Western blot and mRNA

		UPJO	Control	P
IM	P1	2.37 ± 0.96	2.11 ± 1.5	0.31
	P2	2.62 ± 1.95	2.93 ± 2.8	0.30
WB	P1	0.58 ± 0.08	0.57 ± 0.09	0.75
	P2	0.44 ± 0.15	0.44 ± 0.11	0.90
PCR	P1	3.3 ± 1.3	2.6 ± 1.9	0.52
	P2	5.3 ± 2.2	5.3 ± 1.5	0.45

Western Blot

Western blot method was used to examine the expression level of P1 and P2 in UPJO and control groups (Fig. 2). Quantity was calculated the level of protein expression, followed by the calculation of the relative intensity through standardization using the expression of β-actin as the internal control. P1 and was 0.58 ± 0.08 in UPJO and 0.57 ± 0.09 in controls; P2 was 0.44 ± 0.15 in UPJO and 0.44 ± 0.11 in controls. The expression of P1 and P2 between the two group had no statistically significant (P 0.05). Results of the Western blot and P values between the two groups were summarized in Table 1.

Real-time PCR

Amplification and melting curves were drawn according to the mRNA fluorescence values and cycle numbers of P1 and P2, using Beta actin as the internal control (Fig. 3). Each sample was repeated five times in real-time PCR test and averaged as the Ct value. The $2^{-\triangle\triangle ct}$ relative quantitative method was used for mRNA expression analysis by ABI 7500 fluorescence quantitative PCR instrument. The differences in mRNA expression of P1 and P2 in the two groups had no statistically significant ($P < 0.05$) (Table 1).

Discussion

There was a lot of research on molecules mechanisms underlying congenital intrinsic UPJO, but it is still poorly understood (11). Researches demonstrated that a Shh-Ptch-Gli3 dependent mechanism caused murine intrinsic UPJO and implicated dysregulated this signaling in the pathogenesis of human UPJO (5). Shh signaling was consisted of ligands membrane of Ptch receptors, downstream target genes and pathway regulation proteins (4). During renal development, the Shh and its receptor Ptch were expressed in stromal cells and tubular epithelium. Shh bound to Ptch and relieved a transmembrane protein expressed on the cell surface (6). In presence of Shh, GLI proteins (Gli3) translocated to the nucleus, acting as transcriptional activators (12).

It had been previously shown that Shh signaling controlled cellular differentiation during ureter formation. The defection in urothelial differentiation could cause UPJO in mice (13). Previous studies found increased Shh and decreased Gli3 expression (4), Shh might compensatory increase because the downstream target was disturbed. While the Shh receptor was Ptch, the signal was blocked in this phase, so the next downstream target Gli3 was decreased. What had happened in the intermediate passage? We were inconclusive. Recent researches were focused on the Ptch structure (8,11). But it had still not proved that the expression of Ptch (P1 and P2) was abnormal in UPJO. Gupta et al found that the expression of P1 in hydronephrotic model was high as compared to normal mice (10), his research was based on artificial mutant mice, not the real UPJO patients. In Sheybani's research, P1 was increased in a single patient out of eight (5), but the control tissue was contiguous UPJO segment. So, these results could not describe a real pathogenic mechanism underlying congenital UPJO.

Here we obtained 20 intrinsic UPJO tissues and 10 ureters from patients with Wilms tumor, Specimen was strictly collecting and obtaining UPJ segments in two groups. Even so, the results were depressing, our results demonstrated that P1 and P2 were not changed in the quantity of UPJO patients through immunofluorescence, western blot and PCR. We also found that age was not related with quantity of both. All the results were negative and we reached an impasse in our research as well. Previous studies found that P1 was removed from cilia after binding of the Shh ligand to P1 and allowing Smoothened (A G-coupled transmembrane protein) to enter cilia and become activated. If P1 was not removed, it would prevent Smoothened's localization to cilia and disrupt the Gli transcription activator/Gli3 repressor (Gli3R) balance (7). According to this molecular biology, the P1's distribution may be abnormal in the UPJO patients if this pathway had been disturbed. We turned back to examine the immunofluorescence of P1 again, found that red-stained P1 were diffused in control group (Fig. 1B), but were mainly surrounding the nucleuses of smooth muscle cells in UPJO. Based on our previous findings, we inferred that P1's quantity was unchanged, but the movement was restricted in UPJO. Thus, the whole Shh pathway was disturbed. It was only a hypothesis that we inferred from our founds, and it was needed further study to confirm this.

P2 in the shh signaling pathway remains ambiguous. Researches had shown that the two homologs had overlapping functions. But it cannot compensate for the loss of P1 activity (14). Studies found that P2 does not play a functional role in regulating Shh signaling activity during kidney development, but serves as a specific marker of the onset of UPJO (5). In some studies, P2 was increased in UPJO (5), but in our research, P2 was not changed and we could not find any regularities of distribution. We strictly followed the rules of specimen collection of uretero-pelvic-junction in UPJO and control groups, but in other researches, they were not quite exactly the human uretero-pelvic-junction in control group.

Limitations

But we only assayed two factors of P1 and P2. The upstream signal Shh, downstream target factors such as Smoothened and Gli3 were not tested in our research, because they were verified in many studies (3,4). So, our objective was to prove the variation of Ptch. Although we had only seen the abnormal distribution

and unchanged quantity of Ptch. Besides, we found nothing. Next, we will carry further study in interaction between P1 and Smoothened to explore the pathogenesis of UPJO.

Conclusions

The expression of P1 and P2 between the two group had no statistically significant. P1 mainly surrounding the nucleuses of smooth muscle cells in UPJO. The P1 pathway might be disturbed by the abnormal distribution of P1 rather than the quantity.

Abbreviations

P1 Ptch1

P2 Ptch2

UPJO ureteropelvic junction obstruction

Declarations

Authors' contributions: YFY and WWH conceived of the study and performed the experiments and wrote the manuscript. WWH was done the acquisition of data, or analysis and interpretation of date. WPZ participated in the design of the study and provided technical advice. NS was involved in the design of the study, in discussions and reviewed the manuscript. All authors read and agreed to the final manuscript.

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Availability of data and materials: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: The experimental protocol was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University. We obtained all the written consents from the participants or their parents in the study.

Consent for publication: Not applicable.

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

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Figures

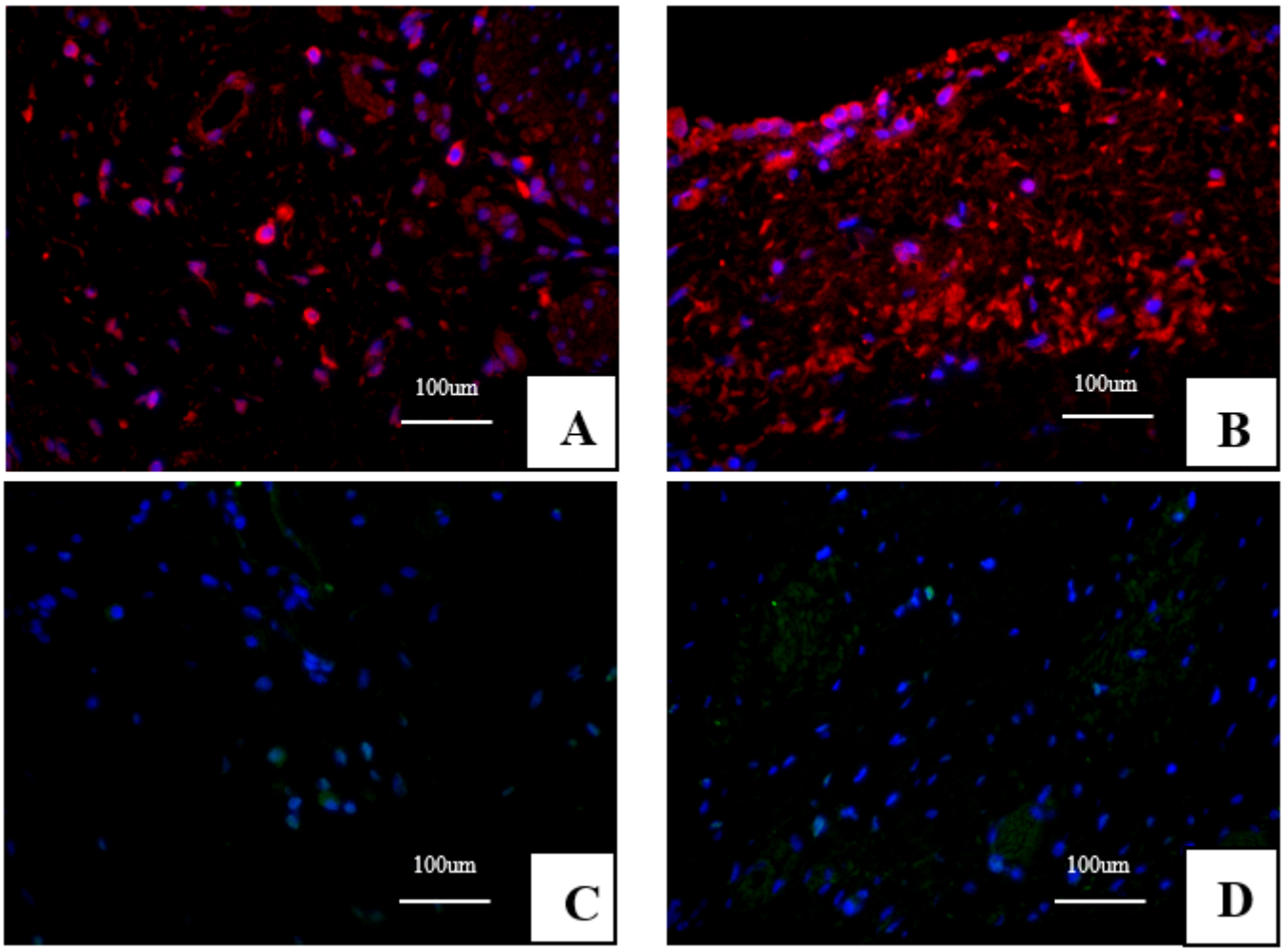

Figure 1

Ptch immunohistochemistry stain (×400). A: P1 was specifically stained red in the stroma in UPJO group. P1's distribution was mainly surrounding the nucleuses of smooth muscle cells (stained blue). B: P1 was stained red and diffused in the stroma in normal control group. C~D: P2 was specifically stained green in the stroma in two groups.

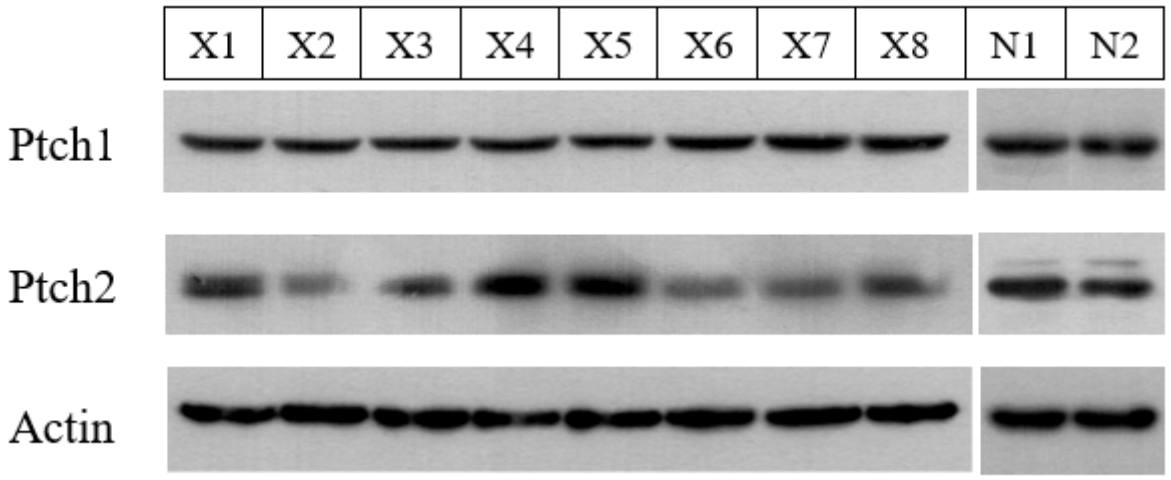

Figure 2

Western blot analysis of P1 and P2 protein expression in UPJO patients and in controls. X indicates UPJO group and N control group. The protein expression of P1 and P2 in UPJO tissue had no statistics significance comparing with control group.

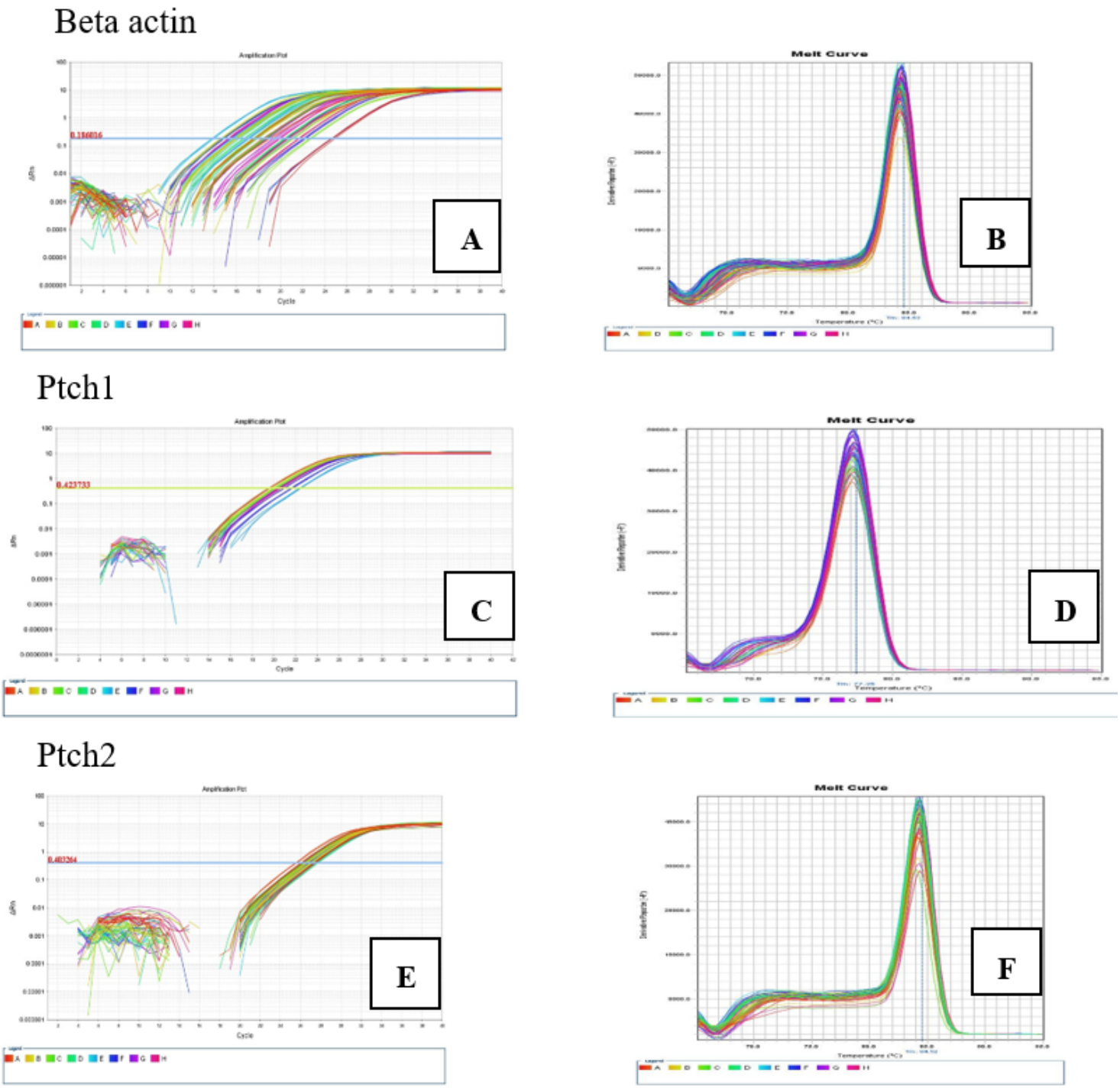

Figure 3

Real-time PCR analysis of 1 and P2 mRNA expression in UPJO and control group. A~B: Amplification and melting curves for Beta actin; C~D: Amplification and melting curves for P1 mRNA expression; E~F:

Amplification and melting curves for P2 mRNA expression.