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Multi-scale mechanical investigation of articular cartilage suffered progressive pseudorheumatoid dysplasia

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Highlights

•The elastic modulus of pseudorheumatoid dysplasia cartilage was first measured.

•Viscoelastic deformation was rigorously considered for accurate measurement.

•Chondrocyte's elastic modulus influenced by pseudorheumatoid dysplasia was reported.

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Abstract

Background

Progressive pseudorheumatoid dysplasia is a rare skeletal dysplasia mainly caused by abnormal autosomal recessive inheritance. Although the main function of cartilage is mechanical support and the characteristics of this disease is the degradation of AC, previous studies on it had been mainly focused on clinical and genetic aspects and the mechanical behavior of the cartilage affected by PPRD is still ambiguous. In this study, we investigate the mechanics and structure of the cartilage suffered disease at multi-scale, from individual chondrocytes to the bulk-scale tissue.

Methods

Depth-sensing indenter were employed to investigate the mechanics of cartilage; we performed atomic force microscope nanoindentation to investigate the cell mechanics and scanning electron microscopy were used to explore the structure feature and chemical composition.

Findings

The elastic modulus of chondrocytes harvested from cartilage suffered from progressive pseudorheumatoid dysplasia is significantly higher than from normal cartilage, same trend were also found in tissue level. Moreover, denser collagen meshwork and matrix calcification were also observed.

Interpretation

The elastic modulus of cartilage should closely related to its denser structure and the calcification, and may potentially be an indicator for clinical diagnosis. The stiffening of chondrocytes during PPRD progression should play a rather important role in its pathogenesis.

Keywords: Cartilage, Chondrocyte, Atomic force microscope, Nanoindentation

1. Introduction

Articular cartilage (AC) provides a smooth, lubricated surface for the load transmission at a low frictional coefficient for absorbing impact energy(Han et al., 2018; Men et al., 2017). The unique mechanical function of AC depends on the composition and structure of the cartilage extracellular matrix (ECM)(Han et al., 2018). The cartilage ECM, consisting of collagen and proteoglycans, is produced and maintained by the chondrocytes surrounding(Li et al., 2018). Under the normal physiological environment, through producing or degrading collagen and proteoglycans, chondrocytes can maintain the dynamic equilibrium of ECM by a balance between catabolic and anabolic activities(Guilak et al., 2018; Guo and Torzilli, 2016). Inversely, biochemical and mechanical properties of ECM will influence the physiological function of chondrocytes(Lofgren et al., 2018). Moreover, the chondrocytes' mechanical behavior will also affect their biological functions, including migration, proliferation and metabolism, and consequently, the influence of the overall mechanical properties of ECM(Moyen et al., 2016; Sliogeryte et al., 2016; Zhou et al., 2014). It is therefore believed that the mechanical properties of ECM and chondrocytes should play an important role in the pathogenesis of cartilage-related diseases, and may be exploited for clinically relevant diagnoses and treatments. Progressive pseudorheumatoid dysplasia (PPRD) first reported in 1980s, is an autosomal recessive inherited skeletal dysplasia with an estimated prevalence of around 1 per million(Chen et al., 2018; Segarra et al., 2012). Its clinical and radiological characteristics, including non-inflammatory, bone-cartilage dysplasia, deformities of multiple joints, muscle weakness, bone pain and cartilage stiffness, have been investigated(Segarra et al., 2012). In 1999, Hurvitz et al. found that the genetic pathogenesis of PPRD should be attributed to abnormal mutations of Wnt1-inducible signaling pathway protein 3 (WISP3) gene which is a member of the connective tissue growth factor (CCN) gene family(Hurvitz et al., 1999). These genes are responsible for encoding proteins involved in cell growth, proliferation and differentiation(Gueugnon et al., 2016; Patra et al., 2016). More cases about WISP3 mutations in PPRD were subsequently reported(Madhuri et al., 2016; Rai et al., 2016; Xiao et al., 2018; Yue et al., 2009; Zhang et al., 2018). However, the mechanical properties of cartilage and chondrocytes under PPRD, which are vitally important in the understanding of the pathology of PPRD, are still ambiguous.

In this study, the mechanical properties of chondrocytes and cartilage suffered PPRD were systematically investigated by atomic force microscope (AFM) nanoindentation and depth-sensing indentation (DSI) at micro and nano meter scale, combining with scanning electron microscope (SEM). It is believed that the presented multi-scale mechanical study on PPRD cartilage should be able to provide useful information that might lead to a better understanding of the pathology of disease and be helpful for the development of novel clinical diagnosis and treatment.

2. Materials and methods

2.1. Cell culture

All experimental produces were approved by the Institutional Ethics Review Committee of the author's institute, and informed consent documents were received from all patients. Fresh knee cartilages were collected from a 12-year-old female who suffered PPRD and OA patients (n = 10; 6 women and 4 men; range 38–79 years old) undergoing total knee replacement surgery. Every cartilage specimen was divided into two groups, one group for

chondrocyte culture, and the other group for indentation and SEM tests. The latter group was immersed in phosphate buffered saline (PBS) and stored in refrigerator (−80 °C) before using. It is difficult to collect completely normal ACs. Therefore, the portions of AC classified as the Outerbirdge Grade 0 from 6 patients were used as the normal group. Chondrocytes were harvested from fresh cartilage following the protocol reported previously(De Palma et al., 2018; Zhuang et al., 2016): in brief, the cartilage was placed in a petri dish filled with phosphate buffer and separated from the bone tissue using a surgeon's knife under sterile condition. The cartilage was cut into small pieces of 3–5 mm3 in size, rinsed with phosphate-buffered saline (PBS) and then digested with 0.25% trypsin (Hyclone) for about 20 min at 37 °C. The digestive fluid was centrifuged, trypsin was removed and then treated with 0.2% type II collagenase (Gibco) at 37 °C for 4 h. After digestion, the cell suspension was filtered with 200 mesh and 400 mesh cell strainers, respectively. The chondrocytes were resuspended in Dulbecco Modified Eagle's Medium (DMEM, Hyclone), containing 10% fetal bovine serum, and cultured in a petri dish at 1 × 105 cells/dish at 37 °C and 5% (vol/vol) CO2.

2.2. AFM nanoindentation

AFM used in this study is JPK NanoWizard II. The tips used for nanoindentation test are CSG10 with nominal spring constant about 0.02–0.05 N/m. Before the AFM nanoindentation, the tips were modified to a flat-ended cylindrical shape with tip radius about 1.0–2.5 µm by focus ion beam (FEI Nanolab650, Holland) (Fig. 1a). The actual spring constant (k) of the cantilever was calibrated using the thermal noise method(Sader et al., 2014; Slattery et al., 2013). The photo-diode sensitivity A was calibrated by performing indentation test on a rigid silicon substrate following the standard calibration procedure(Sader et al., 2014; Slattery et al., 2013).

Fig. 1

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Fig. 1. Typical experimental curves obtained from an AFM nanoindentation test on chondrocytes. (a) The measured PZT movement and the corresponding vertical deflection of cantilever, insert is the SEM image of the FIB-milled AFM tips with flat-ended cylindrical shape; (b) the cantilever deflection versus PZT movement, insert is the microscope image of cantilever and chondrocytes. A significant “load drop” can be observed during the holding segment of PZT movement, this stress relaxation during holding is mainly due to viscoelastic deformation.

The chondrocytes used for the indentation were cultured in a round Petri dish with a diameter of 3 cm. The AFM nanoindentation was achieved by controlling the Piezo electronic transductor (PZT) head's movement: first, moved the tip by moving the PZT head toward the petri dish until their distance was about 300 µm, then shift the petri dish in the same direction by controlling the sample stage to discover the cell cultured on the petri dish. Finally, the indentation test was performed on the central area of the cell and the actual indentation depth was carefully controlled to be within 2 µm. Subsequently, the petri dish was moved toward the same direction until another cell was found, and then the indentation tests were performed on this cell sequentially. 36 PPRD chondrocytes and >250 normal chondrocytes were randomly selected to be measured for each type of cells to ensure that statistically satisfying results could be obtained. It was found that during the

stage holding segment, the vertical deflection that proportional to the load applied on the cell, decreased continually (Fig. 1). This “stress relaxation” is likely to be due to the significant viscoelastic deformation of cells(Zhou et al., 2012). Therefore, we adopted the rate-jump method that can remove viscoelastic effects during measurement for indentation data analysis in this study(Tang and Ngan, 2011; Tang and Ngan, 2012). When applying rate-jump method in AFM nanoindentation, the reduced elastic modulus of cells can be measured using.
(1)
where
is the rate change of the PZT movement just before and after the unloading point and
is the resultant rate change of the recorded photodiode signal D, which is the vertical deflection of cantilever, a is the tip-sample contact radius, which is equal to the tip radius of the AFM tip because the tip used is flat-ended cylindrical. The elastic modulus of cells can be obtained from the calculated reduced modulus by
(2)
where v is the Poisson's ratio. In this study, the Poisson's ratio of the cell is taken to be 0.5(Szymanski et al., 2017). The tip used in this study is made of silicon, which has much larger elastic modulus than those of the cells, therefore the AFM tip can be considered to be rigid body, and Eq. (2) can be rewritten as E = 0.75Er.

2.3. Depth-sensing indentation
Depth-sensing indentation was performed to investigate the elastic modulus and hardness of AC at multi-scale. In addition, another series of depth control indentation was adopted to study the mechanical properties of total OA cartilage tissues with the max displacement distributed in 3000–5000 nm. The indenter is TI 950 Triboindentator supplied by Hysitron company in US, and the indenter tip used is a standard Berkvich diamond tip. During the indentation, the AC tissue was immersed in 1 M PBS to simulate the physiological condition in vivo. Just same as those in AFM nanoindentation, severe viscoelastic effects are discovered in indentation tests results on AC, as verified by the significant “load drop” during the indentation depth holding segment (Fig. 2). Therefore, the unloading rate employed for each loading cycle was very fast, i.e. unload within 0.5 s to minimize the viscoelastic effects during the measurement(Oliver and Pharr, 1992; Oliver and Pharr, 2004). Liquid adhesion was also found during the measurement, as indicated by the negative force observed at the final stage of unloading. Fortunately, as suggested by previous study, in case the sample was fully immersed in the liquid, the negative force caused by the liquid meniscus between the liquid-tip contact would not result in significant errors during the measurement(Tang and Ngan, 2007). The obtained time, load, and depth data were analyzed using the data analysis software integrated in the indenter, which based on the standard Oliver-Pharr model. In the Oliver-Pharr method, the contact depth hc can be calculated from
(3)
where hun and Pun are the indentation depth and load just after unload of each cycle, and S = dP/dh is the contact stiffness just after unload, which can be measured by non-linear curve fitting of the unloading segment. The elastic modulus E and indentation hardness H of the specimen can be calculated from
(4)
and

(5)

Fig. 2
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Fig. 2. Typical step increased depth-sensing indentation results of AC. (a) indentation depth versus time and (b) load versus time. Significant decrease of load was observed during the depth-holding segment, which is the sign of rather severe viscoelastic deformation. Therefore, an ultra-fast unloading rate was adopted, i.e. unload within 0.5 s to minimize the viscoelastic effects on the elastic modulus measurement.

Here Ac is the contact area, which can be directly calculated from the calibrated tip area function Ac = f(hc).

2.4. Structural and chemical analysis

Scanning electron microscope (SEM, TESCAN MIRA 3) was employed to investigate the morphological details of the AC specimens. Before the SEM investigation, the AC samples were washed with deionized water to remove the residual PBS solution. The cleaned samples were then fixed with 2.5% (v/v) glutaraldehyde solution for 30 min(Beyazyildiz et al., 2016). After the fixation, the samples were washed with deionized water for 3 times and dehydrated by 30%, 50%, 75%, 80%, 95%, 100% alcohol respectively. To obtain high-resolution electron images, the fixed samples were coated with a gold layer with thickness around 5 nm using electron sputtering to enhance the electrical conductivity. The chemical content analysis was performed by the energy-dispersive spectrometry (EDS) integrated in the SEM. For each sample, EDS analysis was performed on 6 randomly selected area to exam the chemical compositions.

3. Results

3.1. Chondrocytes from PPRD AC are less deformable than those from normal AC

Chondrocytes are the major cells in AC and respond for collagen fibrils formation and the maintenance of collagen meshwork as well, which is the main solid structural component of AC. The mechanical behavior of chondrocytes will affect their routine biological functions, e.g. migration or proliferation, and consequently, will influence the efficiency of AC generation or reparation(Sophia Fox et al., 2009; Wang et al., 2016). In this study, the elastic modulus of chondrocytes was measured using AFM nanoindentation. The penetration depth of the tip into the cell was controlled in the range of 1–2 µm, and the tip-sample contact area is determined by the cylinder flat-end tip diameter, which is in the range from 1.0 to 2.5 µm. Therefore, we believe that the mechanical responses obtained during the indentation should mainly due to the deformation of the cytoskeleton structure of chondrocyte, which is required to be reshaped continuously during the cell migration.

The elastic modulus of chondrocytes harvested from PPRD and normal AC was investigated using rate-jump method, which are 2.039 ± 0.837 kPa and 0.829 ± 0.456 kPa, respectively (Fig. 3). The standard t-test was performed to analyze the results. We found that the elastic modulus of PPRD chondrocytes is significantly higher than that of normal chondrocytes ($p < 0.001$), this indicated that the chondrocytes harvested from PPRD AC are much less deformable than those from normal AC.

Fig. 3
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Fig. 3. Elastic modulus of chondrocytes measured with AFM indentation using rate-jump method (n = 36). The chondrocytes harvested from PPRD AC are significant stiffer than those from normal AC tissues (n = 10; 6 women and 4 men; range 38–79 years old).

3.2. PPRD cartilage tissue is significantly stiffer than normal AC

The main function of cartilage is mechanical support, for this reason, its mechanical properties play an important role in various joint disorders. In this study, the elastic modulus and hardness of tissue from PPRD cartilage and normal cartilage were investigated at various length scales using depth-sensing indentation. Both the elastic modulus and hardness decrease significantly along with increasing indentation depth, and finally had the tendency to be constant after the indentation depth reach around 2000 nm (Fig. 4). The elastic modulus of PPRD cartilage tissue (179.0–1191.4 MPa) is significantly larger than that of the cartilage from normal tissue (6.1–23.9 MPa). Moreover, it is obviously larger than the elastic modulus of the normal cartilage at 3000–5000 nm, which is mainly distributed between 11.8 MPa and 57.2 MPa. The elastic modulus represents the capability to resist elastic deformation; therefore the current result indicates that PPRD cartilage should be much stiffer than normal cartilage. Similarly, the hardness of PPRD cartilage tissues (10.3–147.6 MPa) is also much higher than normal cartilage tissue (0.3–5.8 MPa), and it is also fairly higher than the hardness of normal cartilage tissue (1.1–9.3 MPa) at 3000–5000 nm, measured by depth control indentation.

Fig. 4
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Fig. 4. The average of elastic modulus and hardness. (a) and (c) are elastic modulus and hardness of PPRD AC and (b) and (d) are normal AC at multi-scale measured using depth-sensing indentation. Both the elastic modulus and hardness of AC tissues decrease with increasing indentation depth and PPRD AC has significantly larger elastic modulus and hardness than normal AC. (e) and (f) are elastic modulus and hardness of normal AC around 3000–5000 nm measured using depth control indentation.

3.3. Collagen meshwork collapses in PPRD cartilage

The basic structural unit of cartilage is collagen fibril, which is consisted of self-assembled procollagen molecules. Individual fibrils are then assembled into larger fibers with diameters ranging from 0.5–3 µm. The microstructures of PPRD and normal cartilage tissue were observed using SEM (Fig. 5). The morphology of the two types of cartilage tissue is significantly different: in the normal cartilage tissue, the diameter of collagen fibers is ~2.0 µm, and the collagen fibers are arranged regularly to form collagen meshwork (Fig. 5c). However, in the PPRD cartilage tissue, it is difficult to spot complete collagen fibers. The tissue stacks to be plate-like structure, and the normal meshwork structure cannot be observed.

Fig. 5
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Fig. 5. SEM images of normal cartilage (a, b and c) and PPRD cartilage (d, e and f). Clear collagen fiber structure can be observed in intact cartilage tissue while in PPRD cartilage, few fibers were observed, the cartilage tissue exhibits plate-like structure.

3.4. PPRD AC has significantly higher calcification level than normal AC

The chemical composition of different AC tissue was investigated using EDS (Fig. 6). Both tissues have carbon and oxygen, which are the main components of collagen, as well as trace elements like strontium (Sr) and sodium (Na). However, the amounts of calcium (Ca) and phosphorous (P) in PPRD AC are abnormally higher than those in normal AC. In PPRD cartilage, the ratio of Ca/P is about 1.55, which is close to the Ca/P ratio of apatite. The EDS results suggested that PPRD AC tissue has been highly mineralized.

Fig. 6

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Fig. 6. EDS spectra of (a) intact normal cartilage tissue and (b) PPRD cartilage tissue, the insets show the detailed distribution of each content.

4. Discussion

Cells are well known to be viscoelastic(Bausch et al., 1998; Guilak et al., 2000; Lu et al., 2006; Tang and Ngan, 2012). The typical “load drop” phenomenon is observed during the AFM nanoindentation tests on chondrocytes (Fig. 1), which should be attributed to viscoelastic stress relaxation(Tang and Ngan, 2011; Tang and Ngan, 2012). The traditional viscoelastic stress relaxation for polymeric materials under room temperature usually takes several hours before the decrease in applied stress can be clearly observed. However, the stress applied on the cell decreased >20% within only 10 s in our indentation tests of cells. It is believed that such rapid and pronounced stress-relaxation behavior should be due to the ultra-small tip radius, which enables much higher stress to be generated, and the viscous fluid nature of cell. It should be noted that severe viscoelastic deformation during nanoindentation test might lead to remarkable errors in measurement, such as Hertz model which based on purely elastic contact assumption(Zhou et al., 2012). We therefore applied the rate-jump method that can eliminate the viscoelastic effects in the indentation data analysis to improve the accuracy of measurement(Zhou et al., 2012). The elastic modulus of chondrocytes from normal AC was measured to be 0.829 ± 0.456 KPa, while the elastic modulus of PPRD chondrocytes was found to be 2.039 ± 0.837 KPa, which is much stiffer than that of normal chondrocytes ($p < 0.0001$, t-test). It should be noted that the elastic modulus measured indeed represents the deformability of the local cytoskeleton, which is closely related to cell's capability for migration. Zhou et al. reported that the elastic modulus of tongue cancer cells is closely related to cells' migration capability, and cells with lower elastic modulus can migrate faster(Zhou et al., 2013). Compared with their counterpart, both the breast and lung cancer cell exhibit lower stiffness, and the lower stiffness lead to the higher chance for migration and metastatic spreading(Plodinec et al., 2012). Therefore, it is believed that the high elastic modulus of PPRD chondrocytes should result in the reduction of their migration capability, and might slow down the cartilage growth and repair.

We also investigated the elastic modulus and hardness of cartilage, which represents the resistance for elastic and plastic deformation respectively, at different length scale using

depth-sensing indentation. It should be noted that the cartilage tissue employed in this study were obtained after the clinical surgery and physiological conditions, which somehow change their original pathological structure. Therefore, we performed the nanoindentation at different locations and only the average of the measured results for further comparison. It was found that both the elastic modulus and hardness for PPRD tissues are significantly larger than those of normal AC. This finding is agreement with the clinical observation of PPRD that the entire tissue becomes stiffer(Sailani et al., 2017; Segarra et al., 2012). The SEM observation and EDS analysis results clearly provide the clues for the stiffening phenomenon observed: there is no complete collagen fibril meshwork can be observed in the PPRD tissues, and the matrix are found to be highly calcified. The disassemble of collagen meshwork will lead to denser tissue structure, which will make the AC tissue stiffer at large length scale(Pang et al., 2017). The significant high calcium content in the collagen fibril indicates the high calcification of matrix, which should be the main reason for the stiffening of PPRD AC tissue at micro- or sub-micro-scale. Another interesting finding here is that both the elastic modulus and hardness of AC tissue decrease with increasing measurement length and this should be due to the hierarchical structure of collagen fibrils, as reported in previous studies(Gautieri et al., 2011).

Chondrocytes will respond to the abnormal mechanical stimuli by remodeling of the cytoskeleton mainly composed of actin filaments and intermediate filaments(Chen et al., 2013). In the PPRD cartilage matrix, the stiffness will increase the stress and strain on the resident chondrocytes, this might trigger the reformation of the cytoskeleton structure of PPRD chondrocytes and then result in the chondrocytes higher stiffness(Blain, 2009). Meanwhile, The stiffness of the surrounding ECM can regulate cell behavior, including proliferation, migration, apoptosis, and differentiation(Nickien et al., 2018). Park et al. found MSCs on soft substrates had less spreading and lower proliferation rate than MSCs on stiff substrates(Park et al., 2011). Allen et al. reported that the differentiation of chondrocytes will be promoted by the suitable ECM stiffness through TGFβ pathway(Allen et al., 2012). In addition, chondrocytes can respond to the mechanical stimuli with changing their anabolic and biosynthetic activity(Carlson et al., 2017). Guilak et al. found that altered loading patterns lead to altered mechanotransduction with downstream cellular signaling which in turn induces cartilage to transform into a degenerative joint disease(Guilak, 2011). In this study, the balance between matrix synthesis and degradation altered due to the increased strain and stress of the cartilage matrix, but how the metabolism changes are desired to be further explored.

In clinical treatment, how to distinguish PPRD patients from osteoarthritis patients is still a difficulty(Taspinar et al., 2016; Wickrematilake, 2017). Recent studies reported that OA AC is around 20–50% stiffer than normal AC(Aigner et al., 2009; Wen et al., 2012), while our depth-sensing indentation results show that the elastic modulus and hardness of PPRD AC tissues are >20 times higher than those of normal AC at different length scale. Therefore, we suggested that the tissue stiffness might potentially be used as an indicator for PPRD diagnosis. Although it is known that the origin of PPRD is known to be the mutation of WISP3 Gene, the detailed pathology is still ambiguous. We believed that our results might capture several unknown frames of the entire pathology (Fig. 7). The stiffening of PPRD chondrocytes origins from genetic mutation will result in the reduction of migration capability of chondrocytes and so the cartilage cannot be efficiently growth or repaired. As a

consequence of the loss of AC repairing ability, the collagen meshwork collapses and the AC tissue becomes denser and stiffer. The dense stiffen AC tissue, on one hand, is not able to effectively absorb the impact energy as normal AC and cause the pain of patient; on the other hand, the higher stress inside denser AC might cause the morphology change and even the apoptosis of chondrocytes, additionally result in disorders of the anabolic and catabolic of chondrocytes, leading to the imbalance furtherly between the synthesis and degradation of ECM.

Fig. 7
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Fig. 7. Schematic to illustrate the possible PPRD pathology. The stiffening of PPRD chondrocytes origins from genetic mutation results in the reduction of their migration capability and therefore the cartilage cannot be efficiently growth or repaired. Consequently, the collagen meshwork collapses and the AC tissue becomes denser. Ca^{2+}gradually diffuses to ECM from subchondral bone, leading to the calcified matrix and stiffer PPRD AC. The denser and stiffer AC tissue, is not able to effectively absorb the impact energy as normal AC and cause the pain of patient; at the same time, the higher stress inside denser AC might cause the morphology change and even the apoptosis of chondrocytes, influence the composition and function of PPRD AC tissue furtherly.

5. Conclusions

In this study, the mechanical and structural properties of PPRD AC tissues and the related chondrocytes were investigated and compared with those from normal AC. It was found that chondrocytes from PPRD AC tissues are significantly stiffer than those form normal AC, indicated the reduction in the capability of migration. Both the elastic modulus and hardness of PPRD AC tissue are much higher than those of normal AC at different length scale, which should be due to the collapse of collagen fibril meshwork and highly calcification of matrix in PPRD AC, the excessive mechanical stimuli might lead to the disorders of the anabolic and catabolic, resulting in the imbalance furtherly between the synthesis and degradation of ECM. Current studies should be able to provide clinical data that will be helpful in PPRD diagnosis and lead to better understanding of the pathology of PPRD, and so facilitate the PPRD clinical treatment.

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Declaration of competing interest

The authors declare no conflict of interest.