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Title: High frequency ultrasound imaging of tidemark ex vivo in advanced knee osteoarthritis

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Abstract

High-frequency ultrasound imaging has been widely adopted for assessment of the degenerative changes of articular cartilage in osteoarthritis (OA). Yet there were few reports on its capability for evaluating subchondral bone. Here, we employed high frequency ultrasound imaging (25 MHz) to examine the cartilage-bone interface *ex vivo* using the cylindrical osteochondral disks harvested from advanced human OA knees, and compare the ultrasound roughness index (URI) derived from the raw radiofrequency signals with micro-CT examination using the Spearman correlation (ρ). URI of the cartilage-bone interface strongly correlated with the bone volume fraction of subchondral plate (ρ = -0.73, p < 0.001) besides bone mineral density (ρ =-0.40, p = 0.020). The increased ultrasound roughness of cartilage-bone interface was in a good agreement with the disruption of tidemark in OA histologically. In summary, we demonstrated that high-frequency ultrasound is a promising imaging tool to evaluate subchondral bone quality and quantity in OA.

Keywords: Osteoarthritis; Articular cartilage; Subchondral bone; High frequency ultrasound; Tidemark; Roughness

Introduction

Osteoarthritis (OA) is a prevalent chronic musculoskeletal disease, which affects millions of old adults around the world. The hallmark of OA includes articular cartilage loss and also subchondral bone disturbance (Li et al. 2013, Wen et al. 2014). Clinically, X-ray imaging is commonly used for the diagnosis of OA and grading of the severity of disease. However, plain x-radiography of OA knee could only depict the macroscopic changes of bone and joint at the advanced stage of disease, such as joint space narrowing, osteophytosis, subchondral bone sclerosis and cystic lesion (Altman et al. 1986). Several MRI techniques have been well developed to assess the morphology of cartilage and bone in OA (Eckstein et al. 2006, Ristow et al. 2009), but the high cost of MRI examination and long scanning time for data acquisition limit its application for screening of knee OA in the community. Arthroscopy is a minimally invasive approach to provide the information about the surface of articular cartilage directly, but it fails to probe the change in either deep layer of cartilage or subchondral bone (Chaturvedi et al. 2017).

Clinical ultrasonography in the range of several MHz can be used for detection of synovitis in arthritic joints; yet it was not sensitive enough to detect the early degeneration of articular cartilage when such a relatively low frequency was chosen (Wang et al. 2010). Considering the limitations of conventional ultrasonography, high frequency ultrasound (normally > 20 MHz) has been adopted to offer a closer inspection of the samples with a much higher resolution. Quite a few studies have showed that ultrasonic signals coming from articular cartilage surface bear the information related to the osteoarthritic change. The ultrasonic parameters such as surface reflection coefficient and roughness index could reflect the quality of articular cartilage and enable us to distinguish the normal and degenerated articular cartilage at its early stage (Brown et al. 2008, Kiviranta et al. 2007, Mannicke et al. 2016, Mannicke et al. 2014, Rohrbach et al. 2017, Saarakkala et al. 2006, Saarakkala et al. 2004, Wang et al. 2010, Wang et al. 2014).

Anatomically, non-calcified articular cartilage is connected with subchondral bone through an osteochondral junction, which mainly consists of an intermediate calcified cartilage layer with two interfaces, i.e. the tidemark on the cartilage side and the cement line on the bone side. Osteochondral junction is most vulnerable for macro- and micro-damages under mechanical stress, which will lead to tidemark disruption, angiogenesis and invasion of sensory nerves and blood vessels from the subchondral bone into the noncalcified cartilage in the initiation of OA (Suri and Walsh 2012). Imaging of the osteochondral junction is desirable for early detection of OA.

Ultrasound has been proposed as a method to assess the cartilage and bone simultaneously (Aula et al. 2010, Brown et al. 2008, Liukkonen et al. 2013, Niu et al. 2012, Saarakkala et al. 2006). The most commonly used parameters include the ultrasound reflection and surface roughness from the two interfaces, i.e., the cartilage surface and the cartilage-bone interface. However, few studies have tried to evaluate the roughness of the cartilage-bone interface and to explore its association with subchondral bone quality and quantity (Table 1).

In this study, we aimed to employ high frequency ultrasound for assessing the cartilage-bone interface using the parameters of ultrasound reflection and roughness. We hypothesized that the ultrasonic changes at the cartilage-bone interface measured by high-frequency ultrasound was closely related to the subchondral bone quality and quantity in the osteoarthritic joints.

Materials and Methods

Sample preparation

Institutional ethic committee approved all the experimental procedures (Ref No:

UW-09368) and informed consent was obtained from each patient in this study. Osteochondral samples were collected from the tibial plateau of 10 patients (3 males, 7 females, age 72 ± 9 years), who received total knee replacement surgery due to late stage of knee OA, from February to April 2016 in one of the authors' institutes. Then there were 3~4 osteochondral disks with a diameter of 10 mm being drilled from each sample, with most of them harvested from the lateral side where more cartilage was remained (Figure 1A&B). A total of 33 osteochondral disks were collected from all the samples and frozen at -80ºC before a series of experimental procedures, including ultrasound, micro-computed tomography (micro-CT) and histological examinations.

Ultrasound Imaging

Osteochondral disks were immersed in the physiologic saline solution, thawed for at least 1 hour, and then fixed at the bottom of a container using some plastic clay (Blu-Tack, Bostik, Thomastown, Australia) for ultrasound measurement (Figure 1C). Radiofrequency (RF) and B-mode ultrasound signals were collected using a linear array transducer (MS550D, VisualSonics, Inc., Toronto, Canada) of a high-frequency ultrasound imaging system (Vevo LAZR, VisualSonics, Inc., Toronto, Canada). Multiple focuses could be set for imaging but for the ease of RF signal processing, a single focus was set in this study, which was placed at the position of the tidemark, i.e.

the second bright line seen in the ultrasound image of the disk. The -3 dB bandwidth was 17 MHz to 33 MHz with a central frequency of 25 MHz for the chosen transducer, which was experimentally determined by measuring the reflected pulse from a polished steel plate. Axial and lateral resolutions of the transducer were 40 μm and 80 μm, respectively, according to information from manufacturer. The transducer could be translated in three directions and rotated when fixed in a positioning system (Figure 1C) and it was adjusted to obtain a maximally reflected signal from the cartilage surface before data collection, indicating an optimized perpendicularity between ultrasound beam and cartilage surface. For spatial averaging, the sample container was horizontally rotated along the center of the disk and the scanning process was repeated at four scans with an angular interval of 45° in the horizontal plane (Figure 1D). Average results from the four scans were used to represent the properties of that sample. Ultrasound signals were digitized as 32-bit floating data at an equivalent sampling rate of 1000 MHz and stored for off-line processing as described in the next part.

Extraction of ultrasound parameters for quantitative analyses

Five parameters, i.e., integrated reflection coefficient (IRC) and ultrasound roughness index (URI) of the cartilage surface, and the tidemark, respectively and cartilage thickness were calculated from the obtained ultrasound signals. Details for extraction of parameters can be found in our previous publications (Wang et al. 2010, Wang et al. 2014) and related calculation methods of thickness, IRC and URI are given in Table 2. In brief, the thickness of articular cartilage was determined by multiplying the time of flight of ultrasound inside the cartilage layer (Figure 2A) by a constant speed of sound of articular cartilage (1620 m/s) (Myers et al. 1995). For IRC, it reflects the strength of ultrasound reflection at tissue interface (Figure 2A), because of acoustic impedance difference on its both sides. The reflection spectrum was firstly corrected by a calibration spectrum measured from a reference steel plate placed at the same distance and then spatially averaged, before finally being averaged within the -3 dB bandwidth to obtain IRC. A window with length of 0.4 μs (about 400 points) was used to gate the signal for spectral analysis. For the tidemark, sample specific reflection at the cartilage surface and attenuation caused by the overlying cartilage layer were also corrected (Saarakkala et al. 2006) using an average attenuation coefficient of 0.27 dB/MHz/mm (Nieminen et al. 2004). For URI, a surface profile was firstly obtained by detecting the surface point from the RF signal, subtracted by its natural curvature, and then a standard deviation was calculated to represent the surface roughness of the interface. A total number of 148 lines were obtained for the scan length of 4 mm so the interval between each two lines was 27 μm. Please be noted that there might be some degradation for the performance of URI measurement at the cartilage surface than the tidemark because the focus was placed at the tidemark interface. Typical images of the disk and the detected interface profiles are shown in Figure 2B. In order to be different from that of the cartilage, a subscription of “bone” was used to indicate the ultrasonic parameter measured from the tidemark. All ultrasound parameters were calculated using custom-written codes using Matlab (V.2014b, The Mathworks Inc., Natick, MA, USA) based on the RF data collected from the osteochondral disks.

Micro-CT examination

Micro-CT was performed to obtain the 3D structure of the subchondral bone for assessing its bone quality and quantity after ultrasound measurement using our established protocol (Wen et al. 2013). In brief, osteochondral disks were scanned by a micro-CT system (VivaCT 40, Scanco Medical AG, Bruttisellen, Switzerland) with an isotropic voxel size of $21 \times 21 \times 21\ \mu m^3$. Bone 3D structures were generated and quantitatively analyzed via the associated micro-CT software (Scanco Medical AG) for both the subchondral plate and subchondral trabecular bone. For the subchondral plate, bone mineral density (BMD), bone volume fraction (BV/TV) and cortical thickness (Ct.Th) were measured. For trabecular bone, the following bone parameters BME, BV/TV, trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and connection density (Conn.D), were measured.

Histological examination

After micro-CT scanning, the samples were decalcified and embedded in wax sequentially for routine histopathological examination using our established protocol (Wen et al. 2013). The samples were positioned along the direction of ultrasound imaging and sectioned at 5μm in thickness for hematoxylin and eosin staining.

Statistical analysis

Non-parametric Spearman correlation was used to analyze the relationship between ultrasound and micro-CT bone parameters and also between ultrasound parameters of cartilage and tidemark. A confidence level of $p < 0.05$ was used to indicate a significant difference or correlation. All the statistical analyses were performed with SPSS (V.21, IBM SPSS Inc., Chicago, USA).

Results

Ultrasound, micro-CT and histological imaging of cartilage-bone interface

As shown in Figure 3, the ultrasound images delineated two interfaces in human knee OA samples. One is the water - articular cartilage surface interface, and the other is the cartilage-bone interface. High-frequency ultrasound images of disintegrated cartilage-bone interface in knee OA samples were consistent with the rough surface of subchondral bone plate in micro-CT images, and also the irregular, discontinuous or double tidemark histopathologically.

Ultrasound roughness index of cartilage-bone interface reflected subchondral bone quality and quantity

IRC were -40.1 ± 3.6 dB at articular cartilage surface and -24.0 ± 7.7 dB at tidemark (the cartilage-bone interface) in all knee OA samples (n = 33). While URI of the cartilage surface and the tidemark was 64.1 ± 25.8 μm, and 36.8 ± 7.4 μm respectively. The thickness of articular cartilage measured under high-frequency ultrasound was 2.66 ± 0.79 mm.

For the correlation analysis, IRC significantly correlated with URI of the cartilage interface (ρ = -0.55, p < 0.001) (Figure 4A). The correlations between the ultrasound and micro-CT parameters were listed in Table 3. Most of the ultrasound parameters of the cartilage interface had no significant correlations with the micro-CT parameters of the subchondral bones, except a weak association between URI and cortical thickness (ρ=0.41, p = 0.017).

For IRC_{bone}, it was found to have no significant correlation with the subchondral plate ($p > 0.05$), but with some of the trabecular bone parameters, including Tr.Sp (ρ = -0.40, p = 0.020), Tr.N (ρ = 0.44, p = 0.011) and Conn.D (ρ = 0.44, p = 0.011). For URI_{bone}, it was significantly correlated to most of the bone parameters, including BMD (ρ = -0.40, p = 0.020), BV/TV (ρ = -0.73, $p < 0.001$) and Ct.Th (ρ = -0.45, p = 0.008) of the subchondral bone plate and BMD (ρ = -0.43, p = 0.012), BV/TV (ρ=-0.39, p = 0.025) and Tb.Th (ρ=-0.52, p = 0.002) of the subchondral trabecular bone. The strongest correlation between ultrasound and micro-CT parameters was found between URI_{bone} and BV/TV of the subchondral bone plate (ρ^2=-0.53, p<0.001) (Figure 4B).

Discussion

The present study adopted two parameters of the second interface (IRC_{bone} and URI_{bone}) derived from the high frequency ultrasound imaging of osteochondral disks for non-destructive evaluation of the microscopic change at the osteochondral junction. It is well known that the reflection of ultrasonic wave from the osteochondral junction was mainly from the interface between calcified and non-calcified cartilage, i.e. the tidemark (Modest et al. 1989). In this sense, both IRC_{bone} and URI_{bone} mainly reflected the changes of tidemark in OA.

Comparisons between previous and present studies were summarized in Table 1. Ultrasound was once proposed for the measurement of subchondral bone in previous studies. In order to penetrate deeper in the bone, an ultrasound frequency as low as 5 MHz was proposed for the measurement of cartilage and bone simultaneously (Aula et al. 2010). The integrated backscattering of the bone, rather than IRC_{bone}, was found in a significant correlation with the bone mineral density of the subchondral plate. Possibly due to poor resolution, URI of the tidemark was not specifically investigated in that study. Compared with previous studies, we adopted the parameters obtained from high frequency ultrasound for the assessment of cartilage and subchondral bone quality in human OA samples. We firstly provided the evidence suggesting URI_{bone} as an indicator for subchondral bone quality and quantity.

Mounting evidence has shown correlations between subchondral bone structure and articular cartilage degradation in early OA using different imaging modalities such as MRI (Bolbos et al. 2008). We also performed the correlation analyses under ultrasound. Compared to IRC_{bone}, URI_{bone} - a morphological parameter of the tidemark was more closely associated with the subchondral bone plate and underneath trabecular bone mass and microstructure although most of the correlation coefficients were weak ($\rho^2 < 0.2$). Particularly, URI_{bone} strongly correlated with the bone quantity (BV/TV) of the subchondral bone plate ($\rho^2 > 0.5$) in addition to weak correlation with the bone quality (BMD) ($\rho^2 < 0.2$). Multivariate regression analysis further proved that BV/TV of the subchondral bone plate was a major independent variable to determine the roughness of tidemark as indicated by URI_{bone} (data not presented here). However, as shown in Figure 4, it was noted that URI_{bone} was actually negatively associated with subchondral plate BV/TV in a non-linear manner. The decrease of subchondral plate BV/TV indicated a loss of structural integrity and an increase of subchondral plate porosity, which might allow new blood vessels and nerves growing and breaching the osteochondral junction in the early stage of OA (Suri and Walsh 2012). Our findings prompt the needs for further investigation into the temporal changes of URI_{bone} in the process of OA development to test whether URI_{bone} could be a robust imaging biomarker for early OA.

Tidemark serves as an interface between the uncalcified cartilage and the subchondral bone. Therefore, the changes of tidemark in OA detected by URI_{bone} might reflect not only the disturbance of subchondral bone but also articular cartilage degradation, particularly that in the deep zone. It has been demonstrated recently that the hypertrophic changes and apoptosis of articular chondrocytes could also be measured based on ultrasound measurement (Rohrbach et al. 2017). Further investigation is in need to look into the weight of bone and cartilage changes that might contribute to the roughness change of tidemark under ultrasonic measurement (Mannicke et al. 2014). More parameters might also be considered in future ultrasound measurement to study different aspects of changes in OA related to various parts of the structure including cartilage, bone and junction.

Originally, we expected that IRC_{bone} might also change with subchondral bone as a result of the acoustic impedance at the tidemark and cement line in the cartilage-bone interface. However, there was a lack of biophysically meaningful and statistically significant correlations between IRC_{bone} and subchondral bone changes in knee OA samples although a few weak correlations were coincidentally found between IRC_{bone}

and trabecular bone parameters (Tr.Sp, Tr.N and Conn.D). The propagation path of ultrasonic wave through the surface and different layers of OA cartilage would affect the calculation of IRC_{bone}. Theoretically, the effect should be precisely corrected yet it was very difficult to measure practically. Taken together, URI_{bone} appeared to be a relatively more reliable imaging parameter for the assessment of tidemark in OA.

There were some major limitations in the current study. Firstly, a fixed attenuation coefficient was used for correction in calculation of IRC_{bone}, which was not precise enough to obtain the true value of this parameter and might affect the practical utility of this parameter. Secondly, the sample number (n = 33) was still quite small, which might not be large enough to generalize our study conclusions. Thirdly, this study was limited to a cross-sectional observation and all of samples were from late-stage of OA knees, which might be the partial reason for a relatively low correlation between measured ultrasound parameters of tidemark and bone micro-CT parameters. It prompts the needs to deploy it in a longitudinal study to generalize our findings. Last but not least, the clinical value of the current imaging approach remains questionable at this stage although it could be employed ex vivo successfully. High frequency ultrasound, while having good resolution, cannot penetrate deep in soft tissue. It remains a major practical issue to be addressed as an in-vivo non-invasive measurement. Intra-articular measurement through an arthroscopic portal using a miniaturized transducer could be an alternative way to identify the microscopic changes at the tidemark in the cases with severe pain but lack of obvious radiological changes (Huang and Zheng 2009, Liukkonen et al. 2014, Viren et al. 2009). However, arthroscopy is still a minimally invasive procedure, which is not appropriate for screening of early OA (Kiviranta et al. 2007).

Conclusions

The feasibility of using high frequency ultrasound imaging for quantitatively assessing osteochondral junctions in knee osteoarthritis has been demonstrated *ex vivo* in this study. The results indicated that high-frequency ultrasound can be a potential tool to measure the morphological (particularly the roughness index) change of the osteochondral junction, particularly the tidemark, as reflection of change of the subchondral bone quality or deep cartilage degradation in osteoarthritis. Future experiment is needed to demonstrate that this method can be used as a clinical tool to measure the change of articular cartilage and subchondral bone simultaneously in osteoarthritis in vivo.

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Figure Captions List:

Figure 1: (A) A schematic diagram showing where osteochondral disks of 10 mm in diameter were extracted for experimental test; (B) A picture of an osteochondral disk with articular cartilage at the top and subchondral bone at the bottom; (C) A schematic diagram showing how an osteochondral disk was positioned for ultrasound measurement and (D) the four scan directions for an osteochondral disk. Please refer to the corresponding text for details.

Figure 2: (A) Left: interactions of ultrasound beam (in red) with the two main interfaces, i.e. the cartilage surface and the cartilage bone interface; Right: typical ultrasound signal of an osteochondral disk where the two echoes from the two interfaces are shown; (B) Left: a typical ultrasound image showing where the two interfaces (in green) are detected; Right: the surface profile signals obtained in ultrasound measurement where ultrasound roughness index can be further calculated.

Figure 3: Typical results for ultrasound imaging (top row), micro-CT (middle row) and histology (bottom row) among different osteochondral disks with different morphologies of the tidemark in human knee OA samples. (A) Smooth tidemark, (B) double tidemark and (C) (D) intermediate levels of tidemark smoothness. Scale bars

indicate a distance of 500 µm.

Figure 4: (A) Spearman correlation (ρ = -0.55) between the cartilage surface roughness (URI) and the integrated reflection coefficient (IRC) from the cartilage surface and (B) Spearman correlation (ρ = -0.73) between ultrasound roughness index of the cartilage-bone interface (URI_{bone}) and the bone mineral density of the subchondral bone plate (BMD_{plate}).

Tables:

Table 1: Comparison between this study and some previous work on using ultrasound for simultaneous cartilage and bone assessment

Study	Specimen, Status	US freq (MHz)	US parameters				Reference method for bone
			Reflection - C	Reflection - CB	Roughness - C	Roughness - CB	
Brown et al. (2008)	Animal, OA	10	√	√	×	×	×
Saarakkala et al. (2006) Niu et al. (2012)	Animal, OA	20 55	√	√	√	×	×
Aula et al. (2010)	Animal, Normal	5	√	√	√	×	pQCT
Liukkonen et al. (2013)	Human, Normal	9	√	√	√	×	µCT
This study	Human, OA	25	√	√	√	√	µCT

Abbreviations: US – ultrasound, C – Cartilage surface, CB – Cartilage-bone interface (tidemark)

Table 2: A list of ultrasound parameters measured from the osteochondral disk

Cartilage thickness	*IRC*	*URI*
$\frac{cT_{tof}}{2}$	$\frac{1}{\Delta f}\int_{f_1}^{f_2} R_c^{dB}\, df$	$\sqrt{\frac{1}{m}\sum_{i=1}^{m}\left(d_i-\bar{d}\right)^2}$

c: speed of ultrasound in cartilage, T_{tof}: time of flight between the two interface echoes

IRC: integrated reflection coefficient, $R_c^{dB}(f)$ is the corrected frequency-dependent reflection coefficient in unit of dB, Δf is the -3 dB bandwidth from $f_1 = 17$ MHz to $f_2 = 33$ MHz. A window with length of 0.4 μs was used to gate the signal at the two interfaces for spectral analysis;

URI: ultrasound roughness index, m = 148 is the total number of points for the surface profile used in the current study, d_i is the surface position at point *i* and $\bar{d}$ is the smoothed surface profile after compensating the natural curvature of the cartilage surface;

Table 3: Spearman correlation (ρ) between the measured acoustic parameters and micro-CT parameters

Acoustic parameters	Micro-CT parameters of subchondral plate			Micro-CT parameters of subchondral trabecular bone					
	BMD	BV/TV	Ct.Th	BMD	BV/TV	Tb.Sp	Tb.Th	Tb.N	Conn.D
IRC	-0.20	-0.10	-0.22	-0.15	-0.15	0.11	-0.02	-0.17	-0.23
URI	0.13	0.18	**0.41***	0.38	0.34	-0.27	0.33	0.33	0.34
Cart. Th	0.01	-0.002	0.29	0.02	-0.02	-0.24	-0.11	0.29	0.28
IRC_{bone}	-0.12	0.07	0.17	0.24	0.25	**-0.40** *	0.10	**0.44** *	**0.44** *
URI_{bone}	**-0.40** *	**-0.73** ***	**-0.45** **	**-0.43** *	**-0.39** *	0.33	**-0.52** **	-0.30	-0.26

Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$