Micro RNA-146a expression, NF-κB/P65 activity and serum pentosidine levels as potential biomarkers for disease severity in primary knee osteoarthritis patients

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KEYWORDS
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Pentosidine;
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Abstract
Introduction: Identifying osteoarthritis (OA) patients at high risk for progression is important.

Aim of the work: To study the expression pattern of micro RNA-146a (miR-146a), NF-κB/p65 binding activity and serum pentosidine levels in patients with primary knee OA (KOA) in order to assess their value as potential markers for disease prognosis and severity and to clarify their role in disease pathogenesis.

Patient and methods: This study was conducted on 36 female patients with primary KOA divided radiologically into those with moderate KOA and severe KOA as well as 20 controls. The expression patterns of miR-146a were analyzed using quantitative real-time-PCR, NF-κB/p65 binding activity and serum pentosidine levels determined using ELISA kits.

Results: miR-146a expression levels were significantly higher in KOA patients than controls being significantly higher in moderate KOA compared to severe cases. NF-κB/p65 binding activity and serum pentosidine levels were significantly higher in severe KOA patients (0.74 ± 0.06 and 425.2 ± 40.3 pg/ml) compared to moderate cases (0.3 ± 0.03 and 311.4 ± 30 pg/ml) (p < 0.05) and were higher compared to controls (0.15 ± 0.08 and 257 ± 32.3 pg/ml respectively) (p < 0.05).

Conclusion: This study may emphasize the role of miR-146a expression, and NFKB/p65 binding activity in primary KOA. Assessment of NFKB/p65 binding activity, miR-146a expression, and serum pentosidine in primary KOA patients could extend the panel of laboratory tests available to monitor the severity and progress of the disease and might benefit as markers for detection of patients with high risk for disease progression; and hence to be a novel therapeutic target to inhibit cartilage destruction.

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1. Introduction

Osteoarthritis (OA) is a complex and multifaceted disease, characterized by the degradation of articular cartilage and joint inflammation [1]. OA is associated with age-related loss of the homeostatic balance between cartilage degradation and repair mechanisms [2]. Several biomarkers are involved in the pathogenesis of Egyptian patients with Knee OA (KOA) as interleukin-1β, tumor necrosis factor-α (TNF-α), matrix metalloproteinase (MMP) [3] and cartilage oligomeric cartilage protein [4]. Vascular endothelial growth factor and osteopontin notably correlated with radiological changes of KOA [5,6]. Understanding the molecular mechanisms involved in the maintenance and destruction of articular cartilage can lead to the identification of novel therapeutic targets for OA.

MicroRNAs (miRNAs) are a type of small, noncoding, single-stranded RNAs that can post-transcriptionally downregulate gene expression [7] and are vital regulatory molecules involved in the pathogenesis of immune and inflammatory diseases [8]. Among these conserved miRNAs, miR-146a is well known for its regulation of the immune response and inflammation [9]. MiR-146a is induced upon the activation of toll-like receptor 4 in the NF-kB-dependent signaling pathway, leading to the down regulation of IL-1 receptor-associated kinase 1 and TNF-receptor-associated factor 6 [10].

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls DNA transcription. It plays a crucial role in immune and inflammatory responses, through the regulation of various genes encoded proinflammatory cytokines, adhesion molecules, chemokines, inducible enzymes and apoptosis [11]. Induction of expression of miR-146a is NF-κB dependent [10]. NF-κB is kept inactive in the cytoplasm by binding to one of the inhibitory proteins called IκB-α, IκB-β, IκB-ε, p105, and p100. As a result of the phosphorylation and subsequent degradation of the inhibitory subunits, NF-κB translocates to the nucleus, binds to κB sites, and regulates target genes. Due to its strong transcriptional activity, the p65 subunit of NF-κB (NF-κB/p65) is responsible for most of NF-κB’s transcriptional activity [12]. NF-κB signaling not only plays a central role in the pro-inflammatory stress-related response of chondrocytes to extra- and intra-cellular insults, but also controls their differentiation program [11].

The incidence of OA increases strongly with age: >50% of the population over 60 years of age shows signs of cartilage damage [13]. One of the major age-related changes in articular cartilage is the accumulation of advanced glycation endproducts (AGEs) [14]. AGEs are modifications of proteins or lipids that are produced through a non-enzymatic reaction between reducing sugars and free amino groups of proteins, lipids, or nucleic acids [15]. Excessive levels of AGEs are pathogenic, resulting in elevated oxidative stress and inflammation [16]. Pentosidine, one of the few chemically characterized AGEs is used as a marker for the process of non-enzymatic glycation.

The aim of this study is to clarify the role of miR-146a gene expression, NF-κB/p65 binding activity and pentosidine serum levels in knee OA (KOA) patients in order to assess their value as potential markers for disease prognosis and severity and to elucidate their role in disease pathogenesis.

2. Patients and methods

This study was carried out on 56 subjects; including 20 healthy females aged 38–58 years representing control and 36 female patients with primary KOA aged 37–63 years presented to Outpatient Clinic of the Physical Medicine and Rheumatology Department, Tanta University Hospitals, Egypt. The diagnosis of KOA was based on the American College of Rheumatology criteria [17]. Those patients were classified according to the Kellgren–Lawrence (KL) grade [18] into Moderate OA (grade 3) (n = 18) and severe OA (grade 4) (n = 18). Approval was obtained from the local research ethics committee, and written informed consent was obtained from each participant.

In all the participants, demographic and clinical information was recorded and body mass index (BMI) calculated. OA patients were subjected to complete history taking including disease onset, and duration and treatment history. Subjects excluded from this study were with other etiologies causing knee arthritis or causing secondary knee OA such as inflammatory arthritis (rheumatoid, gout, pseudo gout), posttraumatic or post septic arthritis, also control subjects who had any signs or symptoms of arthritis or joint diseases.

The severity of OA was evaluated according to the KL grade classification [18] and only patients with KL grades of 2 or higher were included. Participants were radiographically classified into three groups: normal (KL grade 0 or 1), moderate (grade 2) and severe (grade 3 or 4). Controls were defined as not having any radiographic knee OA as indicated by KL grade 0 for both knees.

After 12 h of overnight fasting, 7 ml of venous blood samples was transferred slowly into a dry sterile centrifuge tube, allowed to clot at room temperature, centrifuged at 2000 rpm for 10 min and serum was separated and stored at −70 °C until the time of analysis.

miR-146a expression levels were estimated in peripheral blood mononuclear cells (PBMCs) using real-time PCR. PBMCs were prepared by density gradient centrifugation using Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). Briefly, heparinised blood was carefully layered on Ficoll, and PBMC were harvested from the white interphase after centrifugation for 30 min at 400g, at room temperature and washed with phosphate buffered saline (PBS). The PBMCs samples were stored at −80 °C till the samples were further processed for RNA isolation. Total RNA was isolated from frozen PBMC samples by Qiagen RNeasy Mini Kit according to the protocol supplied by the manufacturer. RNA was eluted and its concentration was measured spectrophotometrically (at 280 nm).

The extracted RNA was reverse transcribed into cDNA using high capacity cDNA synthesis kit, Applied Biosystems. Ten µl of random hexamer primers (Roche, Mannheim, Germany) was added to 21 µl of RNA which was denatured for 5 min in the thermal cycler (Biomera, USA). The RNA-primer mixture was cooled to 4 °C. The cDNA master mix was prepared (5 µl of first strand buffer, 10 mM of dNTPs, 1 µl of RNase inhibitor, 1 µl of reverse transcriptase SuperScript™ II-RT enzyme and 10 µl of DEPC treated water) according to the kit protocol and was added to each sample. The total volume of the cDNA master mix was 19 µl for each sample. This was added to 31 µl RNA-primer mixtures resulting in a reaction volume of 50 µl, which was then incubated in
the programmed thermal cycler one hour at 37 °C, followed by inactivation of enzymes at 95 °C for 10 min, and finally cooled at 4 °C. The RNA was reverse transcribed into cDNA which was then stored at −20 °C. One μl of the cDNA was added to a 20 μl reaction mixture of the QuantiTect SYBR-Green PCR kit (Qiagen) and 0.5 μM from the specific primer pair for human microRNA-149a. This cDNA was then amplified parallel to detect genomic DNA contamination. Primer sequences specific for miR-146a were designed according to Nakasa et al. [19] as follows: sense, 5′-CAG-CTG-CAT-TG G-ATTTAC-CA-3′ and anti-sense, 5′-GCC-TGA-GAC-TCT- GCC-CTT-TG-3′. Primers for U6 snRNA were included as an endogenous control [20] sense, 5′-GCCAGCA-CATATA 3′, and antisense, 5′ TTTACGAATTTGCCGTG- CAT 3′. The determination of the relative levels of gene expression was performed using the cycle threshold (ΔΔCt) method and normalized to the reference gene U6 snRNA.

NFkB/p65 DNA binding activity was estimated in the PBMCs nuclear extracts using transcription factor binding assay kit. Nuclear proteins were isolated from PBMC extract using Nuclear Extract kit (Cat#40010, Active Motif, Carlsbad, CA, USA) according to the protocol of the manufacturer. Briefly, the sample was placed in 0.8 mL of ice-cold hypotonic buffer [10 mmol/L HEPES (pH 7.9), 10 mL KCl, 0.1 mmol/L EDTA, 0.1 mmol/L ethylene glycol tetraacetic acid, 1 mmol/L DTT; Protease inhibitors (aprotinin, pepstatin and leupeptin, 10 mg/L each)]. The homogenates were incubated on ice for 20 min, vortexed for 20 s after adding 50 μL of 10% Nonidet p-40, and then centrifuged for 1 min at 4 °C in an Eppendorf centrifuge. Supernatants were decanted, the nuclear pellets suspended in an ice-cold hypertonic buffer (20 mmol/L HEPES, (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT; Protease inhibitors), incubated on ice for 30 min at 4 °C, mixed frequently and centrifuged for 15 min at 4 °C. The supernatants were collected as nuclear extracts and stored at −70 °C [21]. Concentrations of total proteins in the samples were determined according to the method of Bradford (#Cat No. 500-0006, Bio-Rad Protein Assay) [22].

NFkB/p65 DNA-binding activity was evaluated using the ELISA-based TransAM® NFkB/p65 protein assay (#Cat No. 400096, Active Motif, Carlsbad, CA, USA), following the protocol of the manufacturer. In this commercial kit, a duplexed NF-xb oligonucleotide containing a xB consensus sequence is attached to the surface of 96-well plates. Activated NF-xb dimers in 20 μg nuclear extract bound to the attached oligonucleotide are specifically and quantitatively detected by subsequent incubation with antibodies against the activated forms of NFkB/p65 followed by an enzyme-linked (horseradish peroxidase) secondary antibody for colorimetric scoring [23]. The absorbance was measured on an ELISA reader at 450 nm with a reference wavelength of 655 nm.

Serum pentosidine levels were measured using sandwich ELISA kit (Cat# CEA264Ge, USCNK Life Science Inc., Wu Han, China), according to the manufacturer’s protocol.

Statistical analysis: it was performed using SPSS version 15. Most data are expressed as the mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis was used to compare gene expression levels among three groups. The correlation between the studied clinical and biochemical parameter was calculated using Pearson’s correlation coefficient. p values of <0.05 were considered statistically significant.

3. Results

Comparison among the KOA patients (moderate and severe) and controls regarding age, BMI and studied parameters is demonstrated in Table 1. There were no significant differences between patients with severe and moderate OA regarding age or BMI (p > 0.05). Of note, miR-146a expression levels were significantly higher in patients with moderate compared to those with severe OA (p < 0.05) as shown in Fig. 1. In severe OA, NFKB/P65 binding activity and serum pentosidine levels (0.74 ± 0.06, 425.22 ± 40.28 pg/ml, respectively) were significantly higher than in moderate OA (0.3 ± 0.03, 311.4 ± 30.01 pg/ml, respectively) and in control (0.15 ± 0.08, 257 ± 32.26 pg/ml, respectively) (p < 0.05).

Correlations of the studied parameters with age, BMI and with each other in different grades of OA are presented in Table 2. The relative expression of miR-146a showed a significant positive correlation with age and BMI (r = 0.72 and 0.75 respectively < 0.05), but a significant negative correlation with NFKB/P65 binding activity (r = −0.47, p < 0.05). On the other hand, circulating pentosidine levels exhibited significant positive correlations with age and NFKB/P65 binding activity (r = 0.39 and 0.69 respectively, p < 0.05).

Receiver operating characteristics (ROC) analysis was used to assess the diagnostic value of NFKB/P65 binding activity and serum pentosidine levels and to identify their optimal cut off values. The area under the curve can range from 0.5 to 1 and diagnostic tests that approach 1 indicate a perfect discriminator. The optimal cut off value of NFKB/P65 binding activity was 0.13, the sensitivity at this cut off point was 100%, the specificity was 95%, the positive predictive value was 97.3% and the negative predictive value was 100% and the area covered was 0.95. Serum pentosidine monitoring revealed a sensitivity of 75%, specificity of 100%, positive predictive value of 100%, negative predictive value of 69%, and an accuracy of 0.928 at an optimal cut off value of 300 pg/ml (Table 3, Fig. 2).

4. Discussion

Multiple mechanisms are implicated in the development of primary OA, in which genetic and epigenetic factors appear to interact with environmental factors and age to initiate the disease and stimulate its progression. A potential link between microRNA and several human diseases has been examined [24], however; its contribution to the molecular pathogenesis of primary OA is not fully elucidated. The current study revealed that the expression levels of miR-146a in OA patients are significantly higher compared to controls being highly expressed in early-stage OA which gradually decreases as OA progresses.

In this context, Yamasaki et al. [25] have demonstrated that miR-146 is highly expressed in low-grade OA cartilage, and that its expression is induced by interleukin (IL)-1β stimulation. Synovitis occurs in OA, where circulating PBMCs accumulate in the OA synovium resulting in the production
of pro-inflammatory cytokines and degradative enzymes. Two major cytokines produced by activated synoviocytes, mononuclear cells and articular cartilage and involved in the pathogenesis of OA, are IL-1β and TNFα. These cytokines play a prominent role in OA cartilage degeneration through promoting the release of degradative enzymes such as matrix metalloproteinases (MMPs) and aggrecanases, as well as inhibiting the synthesis of extracellular matrix proteins by chondrocytes. They can also stimulate chondrocytes and synovial cells to produce other cytokines and prostaglandin E2, which in turn diffuse into the synovial fluid thus aggravating joint inflammation [26]. On the other hand, Park et al. [27] proposed that miR-146, which is induced by IL-1β at the early-stage of OA, acts as a negative feedback regulator of the innate immune response by down-regulating two adapter proteins, TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), that are crucial for proinflammatory signaling as they mediate receptor signaling in response to ligands of the TNFα superfamily and inflammatory cytokines (IL-1/Toll superfamily) in knee joint synoviocytes. Furthermore, miR-146a might play a role in repression of catabolic factors as MMP13 and ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin motifs) gene expression in early OA cartilage. Well in line, Li et al. [28] reported that transfection of synthetic miR-146a significantly

**Table 1**  Age, body mass index, nuclear factor kappa B/p65 subunit binding activity and serum pentosidine of the knee osteoarthritis patients (moderate and severe OA) and control.

<table>
<thead>
<tr>
<th>Variables/Groups</th>
<th>Control (n = 20)</th>
<th>KOA patients (n = 36)</th>
<th>F value</th>
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<tr>
<td></td>
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<td>Moderate OA (n = 18)</td>
<td>Severe OA (n = 18)</td>
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<tr>
<td>Age (years)</td>
<td>43.45 ± 8.65</td>
<td>69.11 ± 9.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.44 ± 12.14&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>BMI</td>
<td>22.6 ± 2.42</td>
<td>24.55 ± 3.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.66 ± 5.05&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>NFKB/P65 activity</td>
<td>0.15 ± 0.08</td>
<td>0.30 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74 ± 0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pentosidine (pg/ml)</td>
<td>257 ± 32.3</td>
<td>311.4 ± 30.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>425.2 ± 40.3&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.
BMI, body mass index; NFKB/p65: nuclear factor kappa B/p65 subunit.
<sup>a</sup> Significant at p < 0.05.
<sup>b</sup> Significant as compared to the control.
<sup>bc</sup> Significant as compared to the moderate OA.

**Figure 1** miR-146a expression in KOA patients and controls.
<sup>#Significant compared to control at p < 0.05; @significant compared to moderate OA at p < 0.05.</sup>

**Table 2** Correlation of the studied parameters (miRNA-146a expression, NFKB/P65 and serum pentosidine) with age, body mass index and each other.

<table>
<thead>
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<th>Studied parameters</th>
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<tr>
<td>miR-146a expression with</td>
<td></td>
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<tr>
<td>NFKB/P65 binding activity</td>
<td>−0.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum pentosidine (pg/ml)</td>
<td>−0.12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI</td>
<td>0.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFKB/P65 binding activity with</td>
<td></td>
</tr>
<tr>
<td>Serum pentosidine (pg/ml)</td>
<td>0.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Age (years)</td>
<td>−0.01</td>
</tr>
<tr>
<td>BMI</td>
<td>0.17</td>
</tr>
<tr>
<td>Serum pentosidine (pg/ml) with</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant at p < 0.05.

**Table 3** The performance characteristics for NFKB/P65 binding activity and serum pentosidine between moderate and severe KOA patients.

<table>
<thead>
<tr>
<th>ROC curve between KOA patients and controls</th>
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<tr>
<td>Cutoff</td>
<td>Sensitivity</td>
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<td>-------</td>
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<tr>
<td>NFKB/P65 binding activity</td>
<td>&gt; 0.60</td>
</tr>
<tr>
<td>Serum pentosidine (pg/ml)</td>
<td>&gt; 360</td>
</tr>
</tbody>
</table>

serves as a critical regulator of the inflammatory response, where miR-146a functions to suppress pro-inflammatory signaling by targeting NF-κB transcription factors that are stimulated by pro-inflammatory cytokines, chemokines, stress-related factors, and extracellular matrix degradation products [31]. The present study showed that DNA binding capacity of the p65 subunit of NF-κB was significantly increased in OA patients than controls; being higher in those with severe OA. Preclinical experimental data from animal models of arthritis, including murine type II collagen-induced arthritis and rat adjuvant arthritis, support the essential role of NF-κB on MMP gene expression and the development of inflammatory and histological changes of arthritis [32]. Noteworthy, NF-κB activation in articular chondrocytes regulates the expression of many cytokines and chemokines, adhesion molecules, inflammatory mediators, and several matrix degrading enzymes [33]. NF-κB also influences the regulated accumulation and remodeling of ECM proteins and has indirect positive effects on downstream regulators of terminal chondrocyte differentiation. Thus unlike other signaling pathways the NF-κB activating kinases are potential NF-κB signaling as not only playing a central role in the pro-inflammatory stress-related responses of chondrocytes to extra- and intra-cellular insults, but also in the control of their differentiation program. Thus unlike other signaling pathways keeping NF-κB activation under control can be very important for the design of specific therapeutics [11].

Concomitantly, our data reported a significant negative correlation between miR-146a expression with NFκB/P65 binding activity. MiR-146a has been implicated as a negative feedback regulator of NF-κB activation [8]. Regulation of the inflammatory response via a miR-146a-mediated negative feedback loop is critical for resolution of the NF-κB response in OA. miR-146a is an important check on the amplitude and duration of the NF-κB response. In the absence of this micro-RNA, this response is dysregulated, leading to increased transcription of certain NF-κB-inducible cytokines and chemokine [34].

Nevertheless, our data revealed that serum pentosidine levels were significantly higher in OA patients; being higher in severe than moderate OA patients and exhibited significant positive correlations with age and NFκB/P65 binding activity. This finding is in accordance with that of Senolt et al. [16] who reported increased serum levels of pentosidine in patients with knee OA in comparison to controls, and found a positive correlation between pentosidine levels and cartilage oligomeric matrix protein (COMP), a marker of articular cartilage damage. Along this line, Vos et al. [14] concluded that pentosidine correlated with radiographic progression and thus added to prediction of the burden of osteophyte formation in a cohort of early knee and/or hip OA. Also, Braun et al. [35] showed that the levels of urinary pentosidine are related to the measurements of joint pain, stiffness and disability that were assessed in patients with hand OA.

This finding is biologically plausible as age-associated accumulation of AGE products in joint tissues has been shown to contribute to molecular changes and pathologic alterations in articular cartilage, which then may be more susceptible to damage and development of OA [36]. Pentosidine was characterized as a sensitive marker for all AGEs and was demonstrated to have increased levels in the damaged articular cartilage [37]. Accumulating evidence corroborates the notion that AGEs play a crucial role in the development of OA pathogenesis through several mechanisms. Initially, AGE accumulation makes the tissue more resistant to MMP and thus decreases tissue turnover [38]. Well in line, Rasheed and Haqqi [39] demonstrate that AGEs induce endoplasmic reticulum stress and stimulate the expression of cyclooxygenase-2 through p38-MAPK and NF-κB pathways in human chondrocytes. Alternatively, it has been suggested that AGEs induce Peroxisome proliferator-activated receptor gamma (PPARγ) down-regulation-mediated inflammatory signalings and reduction of collagen II expression in human OA chondrocytes via toll like receptor 4 (TLR4) and receptor for AGEs (RAGE) leading to increased cartilage degradation and chondrocyte hypertrophy in OA cartilage [40]. Of note, our study revealed that diagnostic accuracy of NF-κB/p65 binding activity was superior to that of serum pentosidine levels; suggesting its enormous value as a potential marker for disease progression in OA patients.

In conclusion, this study may provide important insights into the clarification of primary OA pathogenesis; emphasize the role of miR-146a expression, and NFκB/p65 binding activity. The assessment of NFκB/p65 binding activity, miR-146a expression, and serum pentosidine in primary OA patients could extend the panel of laboratory tests available

Figure 2  ROC curves for NFκB/P65 binding activity and serum levels of pentosidine in knee osteoarthritis patients.
to monitor the severity and progress of the disease. They also could be a suitable target for the development of new therapeutic strategies designed to inhibit cartilage destruction in primary OA.

**Conflict of interest**

None.

**References**


