Quantitative analysis of the mRNA expression levels of *BCL2* and *BAX* genes in human osteoarthritis and normal articular cartilage: An investigation into their differential expression

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Abstract. Osteoarthritis (OA) is primarily characterized by articular cartilage degeneration and chondrocyte loss. Although the role of apoptosis in cartilage pathobiology remains to be elucidated, the apoptotic B-cell CLL/lymphoma 2 (*BCL2*) gene family is considered to be involved in OA. The purpose of the present study was to quantitatively analyze the mRNA expression profiles of the *BCL2*-associated X protein (*BAX*) and *BCL2* genes in human OA and in normal cartilage. Cartilage tissue samples were obtained from 78 patients undergoing total knee arthroplasty for OA (OA group) and orthopedic interventions for causes other than OA (control group). Total RNA was isolated from the cartilage tissue specimens and reverse transcribed into cDNA. A highly sensitive and specific reverse transcription quantitative polymerase chain reaction assay was developed for quantification of the mRNA levels of *BAX* and *BCL2*, using beta-2 microglobulin as an endogenous control for normalization purposes. Gene expression analysis was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The mRNA expression of *BAX* presented an increasing trend in the OA group compared with the control group, although without statistically significace (P=0.099). By contrast, the expression ratio of *BCL2*/*BAX* was found to be significantly decreased (2.76-fold) in the OA group compared with the normal cartilage control group (P=0.022). A notable 4.6-fold overexpression of median mRNA levels of *BAX* was also observed in patients with stage III OA compared with the control (P=0.034), while the *BCL2*/*BAX* ratio was markedly (2.5-fold) decreased (P=0.024). A marked positive correlation was observed between the mRNA levels of *BAX* and *BCL2* in the control group (r_s=0.728; P<0.001), which was also present in the OA group, although to a lesser degree (r_s=0.532; P<0.001). These results further implicate apoptosis in the pathogenesis of OA, through molecular mechanisms, which include the aberrant expression of the *BCL2* gene family. Further investigation may reveal novel prognostic biomarkers and potential targets for therapeutic interventions in the early stages of OA.

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Introduction

Although chondocytes account for only 1-5% of the entire cartilage tissue volume, their degeneration contributes to the metabolic and structural changes observed in osteoarthritis (OA) (1-6).

Apoptosis is the genetically regulated form of cell death, which occurs when the cell is exposed to physiological, pathogenic or cytotoxic stimuli, and enables the organism to maintain its homeostasis (7). The susceptibility of cells to apoptosis is regulated by complex molecular signaling systems; proteins encoded by the B-cell CLL/lymphoma 2 (*BCL2*) gene family are major regulatory components of the apoptotic pathway (8-10). The pro-survival family members, including *BCL2*, are integral mitochondrial membrane proteins, which can inhibit apoptosis. The proapoptotic *BCL2*-associated X protein (*BAX*) products, localize to the cytoskeleton in healthy cells, however, following a death signal, they interact predominantly by heterodimerizing with, and inhibiting, the antiapoptotic proteins, thus initiating apoptosis (7,11,12). The expression ratio of *BCL2* to *BAX* appears to be an important determinant of cell susceptibility to apoptosis (9,13)

Cartilage tissue homeostasis is mediated by the resident chondrocytes, and cellular loss leads to the characteristic features of OA tissue, including decrease of cartilage extracellular matrix (ECM) and abnormal tissue remodeling (4,14,15). Due to the chronic nature of OA and the fact that chondrocytes account for little of the total cartilage volume, several studies have reported contradictory results regarding the relative presence of apoptotic cells; thus, the definite contribution of apoptotic cell death in the pathogenesis of the disease is

difficult to determine (4,8,16,17). However, the assumption that cell death is a central feature in cartilage degradation has been examined in various studies, the majority of which report that apoptotic chondrocyte death occurs more frequently in OA than in healthy cartilage (17-23). Furthermore, the cartilage in human OA exhibits nuclear and cytoplasmic features consistent with apoptotic cell death, and the presence of apoptosis in OA cartilage degeneration has been well demonstrated in *in vitro* and *in vivo* models (16,24).

There is increasing evidence that the *BCL2* gene family-apoptotic pathway may be important in the regulation of chondrocyte apoptosis and the aforementioned observed features of OA cartilage degeneration (16,25,26). The mRNA and protein levels of *BAX* and *BCL2* are detectable in chondrocytes of osteoarthritic and normal cartilage. Furthermore their protein levels in OA are reported to differentiate compared with those of the normal cartilage, with differences in their expression patterns between lesional and non-lesional areas of the same osteoarthritic cartilage (16,25,26).

The above-mentioned observations suggest that the relative expression levels of *BAX* and *BCL2* may be a regulator of chondrocyte apoptosis; alterations in the classical apoptotic *BCL2*/*BAX* expression ratio may contribute to the process of cartilage degeneration and may be involved in the pathogenesis of OA. In order to investigate any potential association between the expression profiles of these classic apoptosis-associated genes and the biochemical pathways of OA, the present study quantitatively analyzed the mRNA levels of *BAX* and *BCL2* in normal and osteoarthritic human articular cartilage tissue.

Materials and methods

Cartilage tissue samples. The present study was performed in accordance with the ethical standards set out at the Declaration of Helsinki and was approved by the institutional review board of Attikon University Hospital (Athens, Greece). Patients' written informed consent were obtained prior to the start of the study. Cartilage tissue samples were isolated from 78 patients undergoing orthopedic surgical intervention, which were divided in two groups. The first group was termed the OA group and consisted of 50 specimens isolated from the visibly evident lesions located on the femoral and the tibial articular surface of the knee joint from patients undergoing total arthroplasty for OA. Images of the lesions were captured for documentation and the cartilage specimens were isolated from the lateral compartment of the osteoarthritic knee. The samples were obtained intraoperatively with the use of a sterile scalpel, irrigated with 10cc normal saline, snap frozen and stored at -80˚C until subsequent analysis. The second group was the control group, consisting of 28 tissue specimens that were isolated from non-weight bearing areas of the lateral femoral condyle articular surface during arthroscopic and reconstructive procedures for causes other than OA (n=16), or from visibly healthy weight bearing areas of the lateral tibial or femoral articular cartilage during above knee amputations or joint salvage procedures in patients with non-osteoarthritic knees (n=12). In order to investigate the expression of apoptosis-associated genes in different stages of OA, the radiographic criteria of the Kellgren-Lawrence grading scale (27) were used. According to this classification system, 27 OA samples belonged to patients with stage III osteoarthritis and 23 OA samples belonged to patients with stage IV disease (Table I).

RNA extraction and cDNA synthesis. The osteoarthritic and normal cartilage tissue specimens (~100 mg of each sample) were frozen in liquid nitrogen and pulverized until fine powder was obtained. Total RNA was extracted from the 78 tissue samples using TRIzol reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer's instructions. The resulting RNA pellet was dissolved in a maximum of 5 μl RNA Storage solution (Applied Biosystems/Ambion, Austin, TX, USA) and stored in aliquots at -80˚C until use. The integrity of the RNA was confirmed in randomly selected samples via agarose gel electrophoresis. Following RNA extraction, the total quantity of RNA from each tissue sample was utilized for first-strand cDNA synthesis. The reaction mix also contained 1 μg oligo$(dT)_{18}$ reverse transcription primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each dNTP, 100 units M-MuLV reverse transcriptase RNase H^- (Finnzymes Oy, Espoo, Finland), 20 units RNase inhibitor (HT biotechnology LTD, Cambridge, England) and diethylpyrocarbonate-treated (DEPC) water to a total volume of 20 μl. The reverse transcription reaction was incubated at 37˚C for 60 min and was terminated by incubation at 70˚C for 15 min.

Quantitative (q) polymerase chain reaction (PCR). Gene specific primers were designed and synthesized by VBC-Biotech GmbH (Vienna, Austria) for the amplification of the mRNA transcripts of the *BCL2* and *BAX* target genes, as well as for beta-2-microglobulin (*B2M*), which was used as a reference gene. The previously published genomic sequences were consulted for the design of the primers (GenBank™ accession nos. NG_009361.1, NG_012191.1 and NG_012920.1 for *BCL2*, *BAX* and *B2M*, respectively). *B2M* was selected for normalization purposes due its reported biological stability in human osteoarthritic articular cartilage (28). The *BCL2* specific primers were: forward 5'-TCGCCCTGTGGATGACTGA-3' and reverse 5'-CAGAGACAGCCAGGAGAAATCA-3', producing a 134 base pair (bp) PCR amplicon, the *BAX* specific primers were: forward 5'-TGGCAGCTGACATGTTTTCTGAC-3' and reverse 5'-TCACCCAACCACCCTGGTCTT-3', generating an amplicon of 195 bp and the *B2M* primers were: forward 5'-ACTGAATTCACCCCCACTGA-3' and reverse 5'-AAGCAAGCAAGCAGAATTTGGA-3', resulting in a product of 167 bp.

Reverse transcription-qPCR analysis was performed in 96-well plates on an ABI Prism 7500 Thermal Cycler (Applied Biosystems). The reaction mixture contained 0.2 μl cDNA, 50-75 nM primers, 5 μL Kapa SYBR® Fast Universal 2X qPCR Master mix (Kapa Biosystems, Inc., Woburn, MA, USA), 0.2 μL 50X Rox Low passive reference dye (Kapa Biosystems) and DEPC water in a total volume of 10 μl. The reactions were performed in duplicate under the following conditions: 95˚C for 3 min as a polymerase activation step, 40 cycles of 95˚C for 15 sec for denaturation, and 60˚C for 1 min for primer annealing, extension and fluorescence detection. Each run included a negative control (no cDNA), as well as a

Table I. Characteristics of the normal and osteoarthritic tissue samples.

Characteristic	Cartilage tissue sample, n (%)	
	Normal (n=28)	Osteoarthritis (n=50)
Age (median, mean ± standard error of the mean)	38.5, 40.9±3.4	73.0, 72.7±0.87
Gender (n, %)		
Female	14 (50.0)	42 (84.0)
Male	14 (50.0)	8 (16.0)
Stage of osteoarthritis		
III		27 (54.0)
IV		23 (46.0)

common calibrator sample, which consisted of cDNA reverse transcribed from RNA isolated from the PC-3 human prostate cancer cell line (American Type Culture Collection, Manassas, VA, USA) that was found to be steadily expressed in all genes under investigation, and was therefore used for normalization between the runs. Dissociation curves (60-95˚C, with a heating rate of 0.1˚C/sec) and fluorescence data (every 0.3˚C) were produced following the amplification, in order to corroborate the presence of the predominant reaction products, through their unique melting temperatures (Tm), and the absence of any non-specific products and/or primer-dimers. The Tm of the specific PCR amplicons were, 83.9, 85.7 and 81.3˚C for *BCL2*, *BAX* and *B2M*, respectively. Randomly selected PCR products were also electrophoresed on 3% w/v agarose gels in order to confirm the presence of a unique amplicon.

Calculations were performed using Sequence Detection system, version 1.2.3 computer software (Applied Biosystems). Gene expression analysis was performed using the comparative C_t ($2^{-\Delta\Delta Ct}$) method (29). Briefly, the PCR products were detected by measuring the emitted fluorescence (R_n) at the end of each reaction cycle and average C_T values were calculated for subsequent expression analysis. The threshold cycle (C_t) corresponds to the number of cycles required to detect a fluorescence signal above the baseline. The relative quantification units (RQ units=$2^{-\Delta\Delta Ct}$), representative of the normalized expression of the target genes, were calculated for each sample. $\Delta\Delta C_t$ is the difference between the ΔC_t value of a cartilage tissue sample and the ΔC_T for the calibrator sample, whereas ΔC_t is the difference between the C_t value of the target gene (*BCL2* or *BAX*) and the C_t of the endogenous reference gene (*B2M*).

In order to confirm that amplification was performed with equal efficiencies for the target and the reference genes, thus allowing relative quantification according to the $2^{-\Delta\Delta CT}$ formula, validation experiments were performed using different quantities of input cDNA (Fig. 1). The cDNA dilution series, covering several orders of magnitude, were used for *BCL2*, *BAX* and *B2M* amplification, and the resulting C_t values were plotted against Log10 [cDNA quantity]. The reaction efficiencies (E%) were estimated using the following formula: $E\% = [-1 + 10^{(-1/slope)}] \times 100$.

Statistical analysis. Non-parametric statistical analyses were performed as the distribution of variables between the groups was not Gaussian. For calculation of the *BCL2*/*BAX* ratio, the RQ units of *BCL2* were divided by those of *BAX* for each sample. The Mann-Whitney *U* was used to analyze the differences in the normalized expression levels of *BCL2*, *BAX* and *BCL2*/*BAX* between the groups of individuals. Spearman's correlation coefficient (r_s) was used in order to examine associations between the continuous variables in the investigation. Receiver operating characteristic (ROC) curves were generated for the expression levels of *BCL2*/*BAX* by plotting sensitivity against (1-specificity). The calculations for the Area Under the Curve (AUC) were based on Hanley and McNeil's method (30). To calculate the mRNA expression frequencies of *BCL2* and *BAX* in normal and OA tissue samples, the following cutoff points were used: the 50th percentile (median) for the expression of *BCL2* and the 65th percentile for the expression of *BAX*; differences were assessed using Fisher's exact test. Binary logistic regression models were used in order to estimate the odds ratio (OR) for the presence of OA. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago Il, USA).

Results

Quantitative assessment of the mRNA expression levels of BAX and BCL2. A highly sensitive and specific qPCR assay was developed and evaluated for quantification of the mRNA levels of *BAX* and *BCL2*. The specific amplification of the expected products, according to primer design, was evidenced by a peak in the melting curve analysis and the detection of a single distinctive band in agarose gel electrophoresis for randomly selected cartilage tissue specimens (data not shown). Validation experiments were also performed in order to calculate the qPCR reaction efficiency for each amplicon, using a wide range of inital cDNA template quantities. The amplification efficiencies of *BCL2*, *BAX* and *B2M*, calculated from the slopes of the calibration curves deriving from the validation experiments ($slope_{BCL2}$=-3.446, r^2=0.996; $slope_{BAX}$=-3.479, r^2=0.999 and $slope_{B2M}$=-3.441, r^2=0.998, respectively) were 95.1, 93.8 and 95.3%, respectively (Fig. 1). These data confirmed that the PCR amplicons were produced with similar efficiencies, which consequently enabled the use of the $\Delta\Delta C_t$ method for calculating the RQ expression units of the *BAX* and *BCL2* mRNA transcripts.

Table II. Distribution of mRNA expression levels of *BAX* and *BCL2,* and *BCL2*/*BAX* ratio in osteoarthritic and normal tissues.

Variable	Mean ± SEM	Range	Percentile				
			10	25	50 (median)	75	90
Osteoarthritis (n=50)							
BAX expression[a]	1.64±0.31	0.00426-7.68	0.00426	0.0444	0.739	2.58	5.59
BCL2 expression[a]	118±31	0.342-1,171	0.342	1.04	57.2	132	229
BCL2/*BAX* ratio[b]	208±54	0.0454-1,598	0.840	18.8	61.2	308	467
Normal (n=28)							
BAX expression[a]	0.772±0.258	0.00426-6.39	0.00426	0.0166	0.244	0.870	2.26
BCL2 expression[a]	86.2±26.1	0.342-664	0.947	12.3	41.8	93.9	210
BCL2/*BAX* ratio[b]	418±117	8.38-2,020	33.5	68.4	169	558	1,508

SEM, standard error of the mean; *BCL2*, B-cell CLL/lymphoma 2 gene; *BAX*, *BCL2*-associated X protein gene; [a]relative quantification (RQ) units; [b]*BCL2* RQ units/*BAX* RQ units.

Table III. Frequencies of the mRNA expression levels of *BAX* and *BCL2* between normal and OA tissue samples

Variable	Individuals (n)	Individuals, n (%)		P-value[a]
		Normal	OA	
BAX				
Negative	51	23 (82.1)	28 (56.0)	0.026
Positive	27	5 (17.9)	22 (44.0)	
BCL2				
Negative	39	16 (57.1)	23 (46.0)	0.479
Positive	39	12 (42.9)	27 (54.0)	

OA, osteoarthritis; *BCL2*, B-cell CLL/lymphoma 2 gene; *BAX*, *BCL2*-associated X protein gene; [a]Calculated by Fisher's exact test.

Distribution of the mRNA levels of BAX and BCL2 mRNA and the BCL2/BAX expression ratio in OA compared with normal cartilage. The comparative study of the mRNA levels of *BAX* and *BCL2* between the normal and OA tissue cartilage samples enabled identification of important differential expression patterns. The mRNA levels of *BAX* presented an increasing trend in patients with primary OA (median=0.739 RQ units) compared with the normal individuals (median=0.244 RQ units; Table II), however this was not statistically significant (P=0.099). When the individuals were dichotomized according to the *BAX* mRNA expression status, the expression frequency of *BAX* was significantly higher (P=0.026) in the OA group compared with the normal group (44.0, vs 17.9%, respectively; Table III). The mRNA levels of *BCL2* remained predominantly unchanged (P=0.904) in the OA group (median=57.2 RQ units) compared with the normal group (median=41.8 RQ units; Table II). The expression frequencies of *BCL2* were also similar (P=0.479) between the OA and normal groups (54.0, vs 42.9%, respectively; Table III).

However, the *BCL2*/*BAX* expression ratio was significantly decreased (2.76-fold) in the OA group compared with the normal group (P=0.022), with a median *BCL2*/*BAX* expression ratio of 169 in the normal group and 61.2 in the OA group

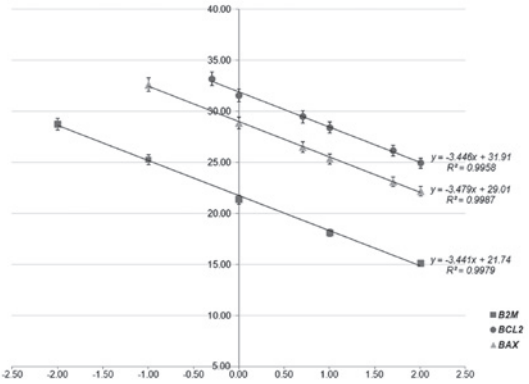

Figure 1. Quantitative polymerase chain reaction for the *BCL2*, *BAX* and *B2M* genes. Calibration curves for the *BCL2*, *BAX* and *B2M* genes. Threshold cycle values were plotted against Log10[cDNA quantity] and data were fit using least squares linear regression analysis. The slopes of the resulting curves were approximately equal, confirming that amplification was performed with similar efficiencies for all three reaction products. *BCL2*, B-cell CLL/lymphoma 2; *BAX*, BCL2-associated X protein; B2M, beta-2-microglobulin.

(Table II; Fig. 2A). Binary logistic regression analysis revealed that individuals with higher *BCL2*/*BAX* expression ratios were significantly less likely to suffer from OA (OR=0.400,

Table IV. Binary logistic regression analysis for the occurrence of osteoarthritis.

Covariant	Crude odds ratio	95% CI	P-value
Log_{10}*BAX*	1.39	0.891-2.16	0.147
Log_{10}*BCL2*	0.828	0.517-1.33	0.432
Log_{10}*BCL2*/*BAX*	0.400	0.181-0.884	0.024
Age	1.29	1.13-1.48	<0.001

CI, confidence interval of the estimated crude odds ratio; *BCL2*, B-cell CLL/lymphoma 2 gene; *BAX*, *BCL2*-associated X protein gene.

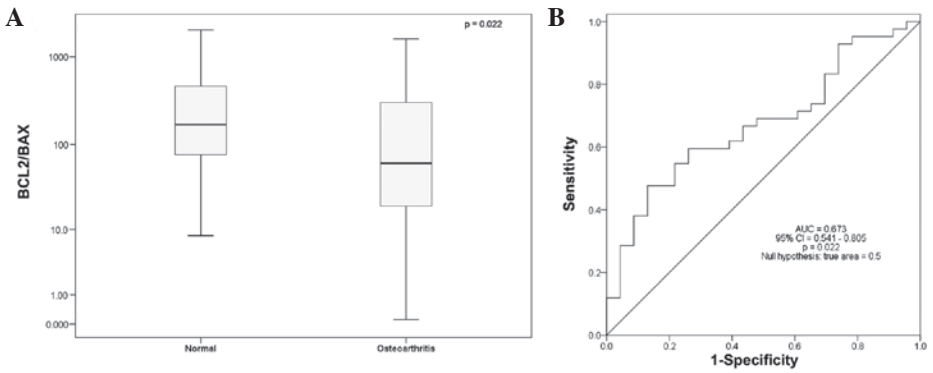

Figure 2. Differential distribution of the *BCL2*/*BAX* expression ratio in normal and OA tissue samples. (A) *BCL2*/*BAX* expression ratio in normal and osteoarthritic cartilage tissue samples. The bold lines represent the median expression, the y-axis is presented in logarithmic scale and the p-value was calculated using the Mann-Whitney *U* test. (B) ROC curve for the *BCL2*/*BAX* ratio. BCL2, B-cell CLL/lymphoma 2 gene; BAX, BCL2-associated X protein gene; ROC, receiver operating characteristic; AUC, area under the curve; CI: confidence interval for the AUC.

95% CI=0.181-0.884; P=0.024), as shown in Table IV. Additionally, ROC curve analysis revealed the important *BCL2*/*BAX* ratio value in effectively discriminating the normal from OA samples (AUC=0.673, 95% CI=0.541-0.805, P=0.022; Fig. 2B).

mRNA expression levels of BAX, BCL2 and the BCL2/BAX ratio in radiographical stages of OA. The expression levels of the apoptosis-associated genes investigated in the present study were also examined at different stages of OA, according to the radiographic criteria of the Kellgren-Lawrence grading scale (27). No statistically significant change was found between stage III and stage IV OA in the mRNA expression levels of *BAX* (P=0.157) or *BCL2* (P=0.395), or the *BCL2*/*BAX* expression ratio (P=0.950).

Notably, a significant 4.6-fold overexpression of median *BAX* mRNA levels was observed between the normal group and the stage III OA group (P=0.034), with the stage III OA group having a median expression of 1.12 *BAX* RQ units, compared with 0.244 in the normal group (Fig. 3A).

In addition, the *BCL2*/*BAX* ratio was markedly decreased (2.5-fold) between the normal group and the stage III OA group (Fig. 3B). The normal group had a median *BCL2*/*BAX* expression of 169, which was significantly (P=0.024) lower compared with that in the stage III OA group (66.8; Fig. 3B). By contrast, the mRNA levels of *BCL2* did not differentiate substantially (P=0.419) between the stage III OA and normal group.

Associations between the mRNA expression levels of BAX, BCL2, BCL2/BAX and patient characteristics. A significant positive correlation was observed between the mRNA levels of *BAX* and *BCL2* in the normal group (r_s=0.728; P<0.001); and in the OA group, although to a lesser degree (r_s=0.532; P<0.001). High mRNA levels of *BAX* were also correlated with increased age, which was statistically significant only for the set of tissue samples derived from normal individuals (r_s=0.502; P=0.007). Similarly, a marked negative correlation was found between the *BCL2*/*BAX* expression ratio and increased age (r_s=-0.580; P=0.004) within the normal group, but not in the OA group. Elevated mRNA levels of *BCL2* were weakly correlated with increased age within the OA group (r_s=0.396; P=0.004), but not among the normal samples. Gender was not associated in any case with the mRNA levels of *BAX* or *BCL2* or the *BCL2*/*BAX* expression ratio (P>0.05, Mann-Whitney *U* test).

Discussion

The hypothesis that chondrocyte cell death is a central feature of the pathogenesis of OA has been examined previously (1,2,22,23). Numerous studies have reported either direct or indirect evidence of increased apoptosis associated with human osteoarthritis (16,24,31-34). *In vitro* and *in vivo* studies have implicated the *BCL2* apoptotic gene family in the regulation of chondrocyte apoptosis and cartilage degradation (16,22,23,25,26).

The majority of studies examining the expression status of the classical *BCL2* and *BAX* apoptotic genes in human OA have demonstrated altered gene expression patterns at the protein level (16,25,26). Due to the limited material often available from cartilage human specimens and the difficulties

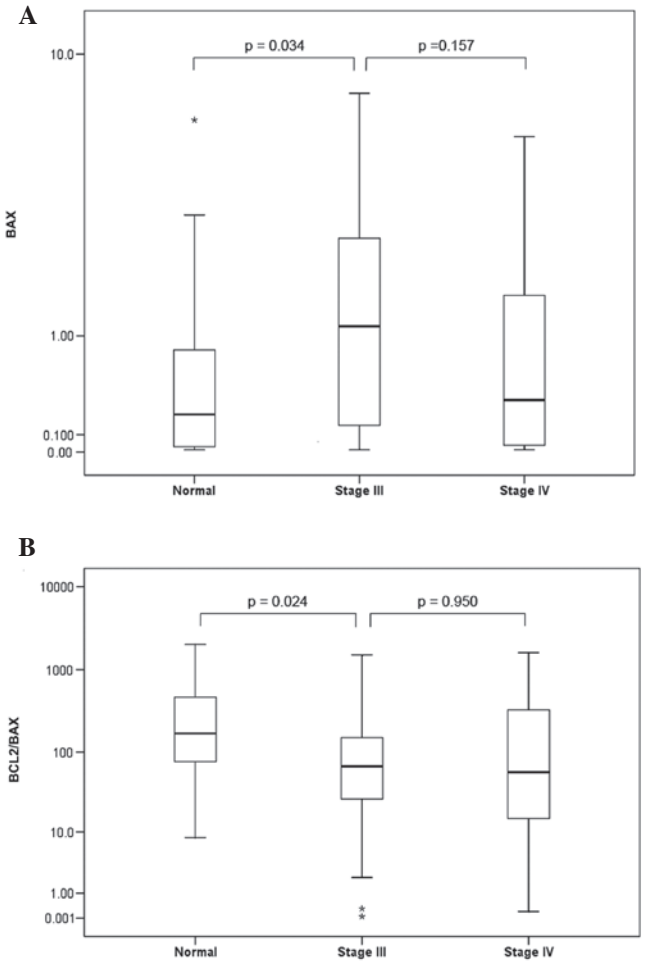

Figure 3. mRNA levels of *BAX* and the *BCL2*/*BAX* expression ratio in normal tissues and OA tissues of different stages of advancement. Distribution of mRNA levels of (A) *BAX* and the (B) *BCL2*/*BAX* ratio in normal, stage III and stage IV osteoarthritic cartilage tissue samples. The bold lines represent the median values and *represent outliers. The y-axis is presented in logarithmic scale. The p-values were calculated using the Mann-Whitney *U* test, by comparing the normal group with the stage III OA and the stage IV OA samples. BCL2, B-cell CLL/lymphoma 2 gene; BAX, BCL2-associated X protein gene.

in isolating total RNA from these samples, there has not been, to our best knowledge, a study that quantitatively analyzes the mRNA expression of apoptosis-associated genes. The present study is the first, to the best of our knowledge, to investigate the differential gene expression of *BCL2* and *BAX* in human OA and normal articular cartilage at the mRNA level, via a hypersensitive and specific qPCR method.

The results of the qPCR demonstrated that the ratio of *BCL2*/*BAX* mRNA expression levels was significantly decreased (2.76-fold) in the OA tissues compared with the normal cartilage tissues (P=0.022). It is well established that a decrease in the *BCL2*/*BAX* expression ratio is associated with the induction of apoptosis in several human tissues, whereas an increase in this apoptotic ratio is associated with a poor prognosis in several types of cancer, and can render tumor cells resistant to the induction of apoptosis by drug therapy (9,13,35,36). Furthermore a study by Chen *et al* suggested that the *BCL2*/*BAX* mRNA ratio is important in governing the susceptibility of human chondrocytes to apoptosis (37).

The gene expression levels of *BCL2* and *BAX* in normal and osteoarthritic tissue samples were also determined in the present study; the mRNA levels of *BAX* in the OA tissues was increased, but without significance, compared with the healthy tissues. However, the expression frequency of *BAX* was significantly elevated in the OA group (P=0.026). This trend was also observed by Hu *et al* in a small sample size of nine OA and six normal cartilage samples, who reported the overexpression of *BAX* mRNA in the OA samples, compared with the normal samples (25). In terms of the gene expression of *BCL2*, no significant difference was observed between the two groups. These results differ from those of Kim *et al*, who reported significantly higher protein levels of *BCL2* levels in normal cartilage, obtained from autopsy, compared with OA cartilage, obtained from patients with knee OA (16). These differences may be attributed to the use of autopsy specimens as healthy controls, since differences in gene expression patterns may be affected by issues of biomolecule stability or alterations in chondrocyte phenotype following death (38).

The importance of the *BCL2-BAX* apoptotic pathway in cartilage degradation was further supported by differences in gene expression levels observed between the normal tissues and different stages of OA, and positive correlations between the mRNA levels of *BAX* and *BCL2* in both the normal and OA groups. Since the OA samples in the present study were obtained from patients during knee arthroplasty, the tissue samples represented only late stages of the disease (KL grading scale III or IV). However, although no statistically significant differences were found between the stage III and stage IV OA samples, a significant (P=0.034) increase in the mRNA levels of *BAX* and a notable decrease in *BCL2*/*BAX* ratio were observed between the normal samples and stage III samples (P=0.024). These observations suggest that a balance between the gene expression levels of *BCL2* and *BAX* is required to maintain tissue homeostasis and, when this balance is disturbed, the cell response to apoptotic stimuli may drive the progression of OA.

A correlation between the expression of classical apoptotic genes and patient age was also observed. A positive correlation between the mRNA levels of *BAX* and increasing age, and a negative correlation between the *BCL2*/*BAX* ratio and increasing age were observed. Several studies have implicated the expression levels of *BCL2* and *BAX* in the molecular basis of age-related changes in several human tissues (39-43). In a comparative study of a mouse OA model Mistry *et al* reported that the protein levels of *BCL2* and *BAX*, analyzed by immunohistochemistry, decreased with age (44). In the present study, the groups were not age-matched, since the prevalence of OA in young individuals is low, and obtaining healthy knee cartilage specimens from aged individuals is extremely difficult (38). The association between the qualitative and/or quantitative differences in the extracellular matrix of the articular cartilage and aging may contribute to the reduction in the maintenance and repair potential of old cartilage and the increased incidence of degenerative cartilage disease (4,45-47). However, whether alterations in the differential expression patterns of *BCL2* and *BAX* contribute to the vulnerability of aging cartilage, or vice versa, during the development of OA remain to be elucidated. Further investigation is necessary to identify whether the alterations in the relative gene expression levels

of the *BCL2*/*BAX* apoptotic pathway reflect the response of chondrocytes to the increased biomechanical load, decrease of the cartilage repair potential due to the normal aging process or the severity of OA.

Although the role of apoptosis and of the *BCL2*/*BAX* gene apoptotic pathway in the development of OA remains to be fully elucidated, delineation of the apoptotic mechanisms occurring in the articular cartilage tissue may offer potentially useful therapeutic targets for OA. Chen *et al* reported that selenium partly inhibits the apoptotic cell death induced by T-2 mycotoxin in human chondrocytes by decreasing the *BCL2*/*BAX* ratio (37). Feng *et al* reported that chondrocytes overexpressing *BCL2* are resistant to apoptosis induced by serum withdrawal and retinoic acid treatment (48), while Mukherjee *et al* reported that staurosporine-mediated chondrocyte death coincided with increased mRNA expression levels of *BAX*:*BCL-X*, and that pretreatment of cultures with nimesulide or ibuprofen, protects chondrocytes against cell death (49). Furthermore, Amling *et al* demonstrated that PTHrP stimulates the protein expression of *BCL2* in chondrocytes *in vivo* and *in vitro* (50). Further investigations may reveal whether the pharmacological inhibition of cell death in chondrocyes is clinically valuable for OA (4).

Despite increasing evidence that chondrocyte apoptosis is associated with the development of OA, the importance of that link, and whether chondrocyte apoptosis is a cause or a result of cartilage degeneration, remains to be elucidated (2,51). The results of the present study further implicate apoptosis in the pathogenesis of OA, through molecular mechanisms, which include the aberrant expression of the *BCL2* gene family. Additional investigation of this association, and the ability to intervene in the process of apoptosis may reveal novel prognostic biomarkers and potential targets for early therapeutic interventions in the treatment of OA.

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