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Short Communication

Identification, Characterization and Expression Analysis of *Biglycan* in Asian Elephant *(Elephas maximus)* [1]

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Abstract

The aims of this study were to investigate the coding sequence and the deduced amino acid sequence of Asian elephant's biglycan gene as well as its expression in different tissues and conditions using wound healing as a model. The results showed that Asian elephant biglycan coding sequence was 1,110 base pair (bp) long (accession number: JQ753329), encoding 369 amino acids. The coding and amino acid sequences between Asian and African elephants revealed 99% and 98% similarity, respectively. The conserved domains of biglycan protein were also observed. In addition, its expression was found in 15 tissues with a predominant expression in cartilage and spleen. For expression analysis in the wound healing process, it was found that the level of biglycan mRNA was influenced by many factors, including age, type of wound and stage of wound healing.

Keywords: *Asian elephants, Biglycan, Gene expression, Sequencing, Wound healing*

Asya Filinde *(Elephas maximus)* Biglikanın Tanımlanması, Karakterizasyonu ve Ekspresyon Analizi

Özet

Bu çalışmanın amacı Asya filinde biglikan kodlayan sekans ve sonuçlanan amino asit sekansı ile farklı dokulardaki ekspresyonunu araştırmak ve yara iyileşmesindeki durumunu incelemektir. Elde edilen sonuçlar Asya fili biglikan kodlayan sekansın 1110 baz çifti (Ulaşım Numarası: JQ753329) olduğunu ve 369 amino asit kodladığını göstermiştir. Asya ve Afrika filleri arasında kodlama ve amino asit sekansları birbirleriyle sırasıyla %99 ve %98 oranında benzerlik gösterdi. Biglikan proteinde korunmuş domainin varlığı gözlemlendi. Ayrıca biglikan ekspresyonu 15 farklı dokuda tespit edilirken kıkırdak ve dalakta baskın olarak eksprese edildiği belirlendi. Yara iyileşmesindeki ekspresyon analizinde biglikan mRNA seviyesinin yaş, yara tipi ve devresi gibi birçok faktör tarafından etkilendiği tespit edildi.

Anahtar sözcükler: *Asya fili, Biglikan, Gen ekspresyonu, Sekanslama, Yara iyileşmesi*

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INTRODUCTION

Asian elephants (*Elephas maximus*) have a close relationship with Thais and their culture for more than 700 years. However, Asian elephants became an endangered species in the International Union for Conservation of Nature (IUCN) Red List and the first account of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Health problem is an important factor influencing their life quality [1], especially diseases or disorders in musculoskeletal system and wounds [2]. Although many clinical studies have been done on musculoskeletal system and wound healing of Asian elephants, little is known about the musculoskeletal genes of this species.

Many genes involving musculoskeletal system have been identified and characterized. Biglycan, also known as BGN, PG-1, DS-PG1, and PG-S1, is a member of the small leucine-rich proteoglycan (SLRP) family. This proteoglycan is associated with extracellular matrix (ECM) formation as an important structural component and signaling molecule [3], which can be found in many organs [4]. Therefore, the alteration of biglycan at DNA, RNA and protein can influence the progression and the recovery of many diseases and disorders, such as degenerative joint disease, chronic heart failure and other inflammatory diseases [5-7]. The *biglycan* gene has been identified in many organisms, but, for Asian elephant, only the intron region of the gene was studied. In addition, there was no previous report on its gene expression profile in various tissues of the Asian elephant, which is generally studied together to indicate the gene function and its expression pattern on pathological conditions. Hence, this study was conducted to investigate the coding sequence of the *biglycan* gene, the mRNA expression of this gene in the various tissues of the Asian elephant including its expression pattern in some pathological conditions.

MATERIALS and METHODS

This research consists of two main studies. The first is the identification and characterization of coding sequence of the *biglycan* gene and its deduced amino acid sequence in Asian elephant. The other study is to provide the expression levels of *biglycan* gene in various tissues of Asian elephant, including tissues undergoing wound healing at different conditions as an example model.

Animals

Different tissues (skin, pancreas, heart, cartilage, large intestine, kidney, lung, muscle, spleen, liver, cecum, lymph node, small intestine, placenta, thymus) were collected from six Asian elephants (age of 1 day to >60 years) immediately after death. Wounded skin samples were taken from three living elephants with different wound conditions from the Thai Elephant Conservation Center (TECC) (Lampang, Thailand) and the Friends of the Asian Elephant Organization. The information regarding the Asian elephants is provided in *Table 1*. These tissues were stored at -80°C until use.

RNA Isolation and Reverse Transcription

Total RNA was extracted from 20 mg of tissue samples using InviTrap® Spin Universal RNA Mini Kit (Invitek, Germany), according to the manufacturer's protocols. The contaminated genomic DNA was eliminated using DnaseI (Fermentas, USA), following the manufacturer's instructions. The RNA quantity, purity, and integrity were verified using both native RNA electrophoresis on 1% agarose gel and UV absorbance ratio at 260 nm and 280 nm. cDNA was synthesized from 100 ng of total RNA using M-MuLV® Revertid reverse transcriptase (Fermentas, USA) at 65°C for 5 min, 37°C for 5 min, and 42°C for 90 min in Thermal Cycler (Biorad, USA).

Experiment 1: Identification and Characterization of *Biglycan* Coding Sequence

Cloning and Sequencing of Coding Sequence of Asian Elephant Biglycan cDNA

The first partial coding sequence of *biglycan* was amplified using primer pair (F1/R1). This primer pair was firstly designed from the conserved regions of various mammals including cattle (NM_178318), pigs (XM_003135475), mice (NM_007542), rats (XM_001057996), orangutans (NM_001132116), dogs (NM_001003229), rabbits (NM_001195691), and sheep (NM_001009201). The partial coding

Table 1. *Name and description of Asian elephants used in this study*

No.	Name	Age (years)	Sex	Sampling Tissues
				Gene Expression Analysis in Various Tissues
1	Bua-ngern	50	F	skin, pancreas, heart, cartilage, large intestine, kidney, lung, muscle, spleen, liver, cecum
2	Kod	>65	M	skin, heart, large intestine, kidney, lung, spleen, liver, small intestine
3	Somjai	60	F	cartilage, kidney, spleen
4	Thongtae	3	M	heart, lung, spleen, liver, lymph node, small intestine
5	Baby	1 day	M	heart, kidney, spleen, liver, lymph node, thymus, placenta
6	Lomsak	2	M	heart, kidney, small intestine
No.	**Name**	**Age (years)**	**Sex**	**Gene Expression Analysis in Wound Healing**
7	Momae	18	F	nearly closed, acute wound on left foot
8	Mogradee	19	F	new, acute wound at belly
9	Saithong	35	F	chronic wound on forehead

sequence of *biglycan* was then cloned into the TA cloning vector (RBC Bioscience, Taiwan), and sequenced by 1st BASE, Malaysia. The obtained partial sequence was then used together with African elephant *biglycan* gene (XM_003421701.2) to design the F2/R2 and F3/R3 primer pairs, to amplify the whole coding sequence. Next, the *biglycan* fragments amplified from primer F2/R2 and F3/R3 were also cloned and sequenced. The coding sequences were acquired from three individual Asian elephants.

Nucleotide and Amino Acid Sequence Analysis

Three partial coding sequences were analyzed using the BLAST program [8] and combined into a complete *biglycan* coding sequence by Clustal X program [9]. The amino acid sequences were deduced using the six-frame translation program (www.bioline.com/media/calculator/01_13.html) and analyzed by the BLAST program.

Experiment 2: Gene Expression Analysis of Biglycan in Asian Elephant

Expression Analysis of Asian Elephant Biglycan mRNA Expression in Different Tissues and Wound Healing Using Real-time PCR

The mRNA expression of *biglycan* in different samples was performed using Real-time Thermal Cycler, MyiQ5 (Biorad, USA) with 2X SYBR Green qPCR Master Mix (RBC Bioscience, Taiwan), following the manufacturers' instructions. The primer pair F1 and R4 (5'-CAGGTTCAA AGCCACTGTTCTCC-3'), which was designed based on the newly discovered *biglycan* coding sequence, was utilized in this experiment. The housekeeping gene, *glyceraldehydes 3-phosphate dehydrogenase* (*GAPDH*), was used as the internal normalization with the GAPDH primer (F: 5'-ATC ACTGCCACCCAGAAGA-3', R: 5'-TTTCTCCAGGCGGCAGGT CAG-3') designed from the accession number, FJ423089.1. PCR reaction was performed at 95°C for 5 min; 45 cycles of 95°C for 15 s, 62°C for 30 s, 72°C for 30 s, and, 72°C for 7 min. The expression of *biglycan* was calculated by the $2^{-\Delta CT}$ method and normalized by the *GAPDH* expression [10].

Statistical Analysis

Statistical analysis using student's t-test and analysis of variance (ANOVA) was conducted to determine the difference in the mean values of the expression level between groups of the different tissues and mRNA levels of individual wounded-skin samples, respectively. The *P* value for significance was set at $P \leq 0.05$.

RESULTS

Identification and Characterization of the Coding Region of Asian Elephant Biglycan Gene

The full length of *biglycan* cDNA of the Asian elephant was amplified, found to be 1,110-bp long *(Fig. 1)* and deposited in the GenBank database as accession number, JQ753329. The sequence shared 99% homology with that of African elephants. Among the three Asian elephant *biglycan* sequences derived in this study, a single nucleotide polymorphism (SNP) at position 303 was exhibited as thymine (T) or cytosine (C), resulting in a silent mutation which was translated to asparagine *(Fig. 1)*. The 369-amino-acid sequence of Asian elephant *biglycan* in the primary structure was presented in *Fig. 1*. The putative molecular weight was 3.7 kDa with an isoelectric point of 8.39. This amino acid sequence also shared 98% homology with that of the African elephant.

Expression Analysis of Asian Elephant Biglycan in Different Tissues Using Real-time PCR

It was found that the Asian elephant *biglycan* mRNA was ubiquitously expressed in different levels in various tissues *(Table 2)*. High levels of expression in cartilage and spleen were observed, while the *biglycan* mRNA levels in skin, pancreas, muscle, cecum, and placenta were relatively low (expression level <0.1). Significantly higher expression level of *biglycan* mRNA was found in young elephant group (1 day - 3 years), compared to those of the old group (≥50 years), in spleen, liver and small intestine. But in kidney, lung and heart, there was no significant difference between the two groups. For gene expression in wounded skin at different pathologic conditions, the result was showed in *Table 3*.

DISCUSSION

Due to the close relationship in evolution between the Asian and African elephants as they belong to the same order, Proboscidae, we hypothesized that both coding sequence and amino acid sequence of biglycan of the Asian elephant might be similar to those of the African elephant. The 99% and 98% homology of the biglycan coding and amino acid sequence, respectively, between the Asian and African elephants agreed with our hypothesis. Six conserved structures including signal peptide sequence, pro-peptide region, LRR regions, Cysteine loop (CX_3CXCX_6C) at N-terminal that defines it as a member of SLRP classI, Cystein loop at C-termnial and Ser-Gly dipeptides, the GAG attachment site, were also observed and similar to the structure of biglycan in other species [4,11]. These similar structures may suggest the function of biglycan as an important component for connective tissue formation [5,6,11].

This study was the first report of *biglycan* gene expression profile in various tissues of the Asian elephant. From the result, *biglycan* mRNA was predominately expressed in cartilage and spleen. This was consistent with the pre-vious study in other species [12,13]. Moreover, the decreased expression level of *biglycan* in spleen, liver and small intestine in the old elephant group was found.

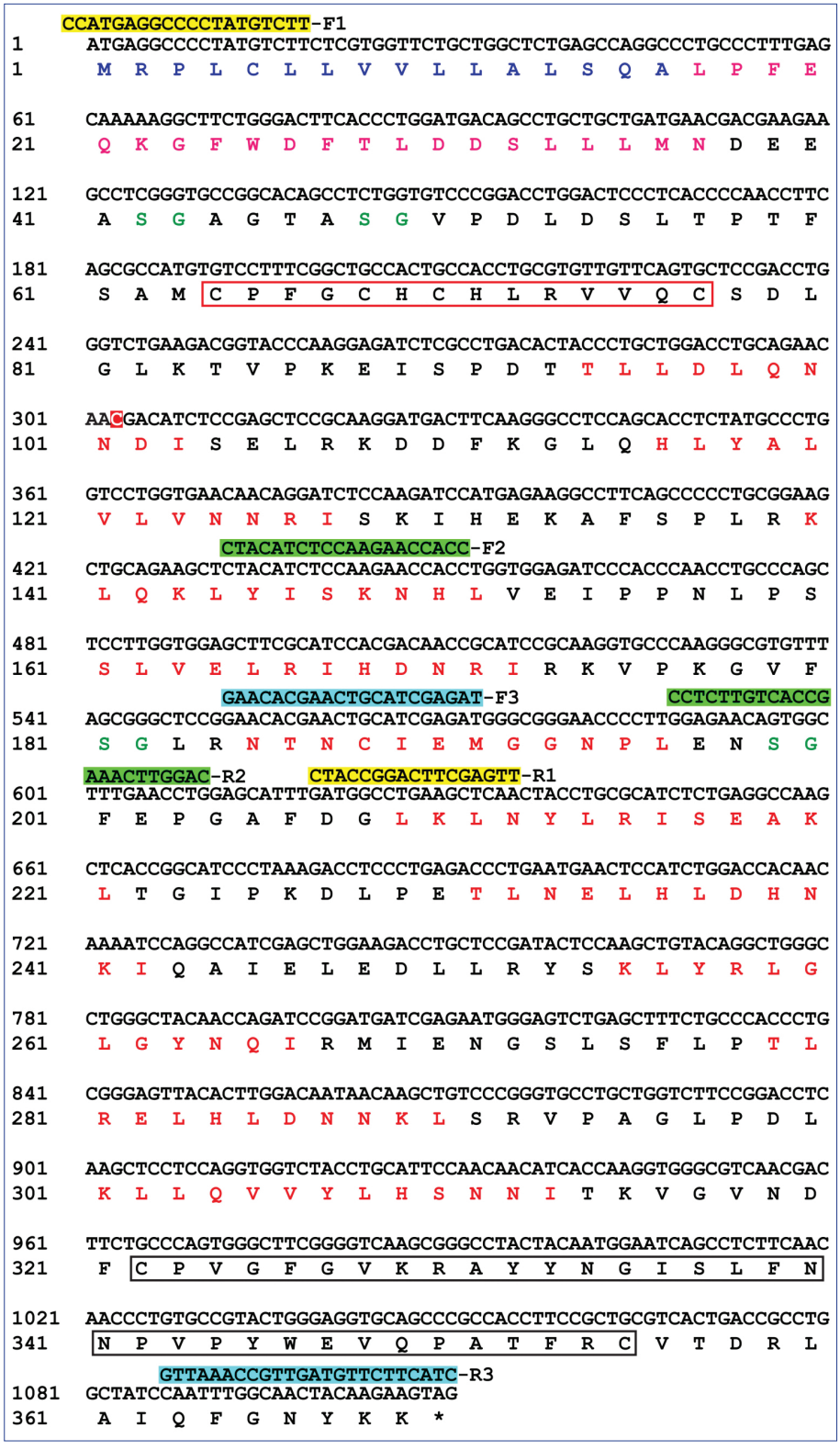

Fig. 1. The nucleotide and deduced amino acid sequence of Asian elephant biglycan including the primers, and their binding sites, used to clone this coding sequence. The primer sequence with its name were shown above the nucleotide sequence with highlight. Forward primer sequences are given in 5′ to 3′ direction. Reverse primer sequences are shown as 3′ to 5′ direction. The red highlight with white letters indicates a nucleotide with polymorphism (T or C). The asterisk shows a stop codon as TAG. The blue and pink letters indicate the putative signal peptide (amino acid 1-16) and pro-peptide region (amino acid 17-37), respectively. Four Ser-Gly dipeptides are indicated by green letters. The Cystein loop at N-terminal (C^{64} to C^{77}) is given in red box. Ten LRR regions (x-L-x-x-L-x-L/I-x-x-N-x-L/I) are indicated by red letters. The letters in black box represent the Cystein loop at C-terminal (C^{322} to C^{355})

This was similar to the decline of *biglycan* mRNA in human articular cartilage which correlates to the increasing age [14]. Although the difficulty in acquiring normal sample was a critical factor that affected the limited expression profile of the gene as shown in *Table 2*, these data could support the function of biglycan and indicated the preliminary trend of its gene expression pattern in various tissues of the Asian elephants.

Skin wound was chosen as a model in this study due to its simplicity of sample collection and observation of the pathophysiology of the samples. Because age and gender are the important factors that affect the physiological changes of wound healing [15]. The significant difference of *biglycan* expression between Momae and Mogradee (similar age and same sex) might be due to the different stage of wound healing process, as Momae had the nearly closed wound (maturation stage) but Mogradee had the open wound with pus (inflammation and proliferation stages). The higher expression of *biglycan* gene in Mogradee, compared to that of Momae, might be a result of its function as an early response gene in inflammatory condition and a participating gene in ECM formation during the proliferation stage [3,7]. For Saitong, the lowest expression level might be due to the older age of Saitong than those of the other elephants. Besides, loss of growth factors in chronic wound [16] especially transforming growth factor-β (TGF-β) might be a critical factor for the low expression of *biglycan* that is usually stimulated by TGF-β in ECM formation [14]. Thus, the expression pattern of *biglycan* mRNA in wound healing in Asian elephant in this study was influenced by age, stage of wound healing and wound type, which was similar to the previous studies [5]. This was also in agreement with

Table 2. *The relative expression of Asian elephant biglycan gene between young and old groups in various tissues*

Tissues	Relative expression	
	Young Group	Old Group
Skin	NS	0.007±0.002
Pancreas	NS	0.038±0.018
Heart	0.134±0.062	0.361±0.282
Cartilage	NS	2.085±0.437
Large intestine	NS	0.103±0.034
Kidney	0.038±0.012	0.142±0.181
Lung	0.260±0.004	0.168±0.115
Muscle	NS	0.003±0.002
Spleen *	3.072±1.138	0.313±0.206
Liver *	0.474±0.377	0.024±0.011
Cecum	NS	0.042±0.007
Lymph node	0.294±0.251	NS
Small intestine *	0.964±0.538	0.101±0.014
Placenta	0.016±0.002	NS
Thymus	0.267±0.048	NS

The data are represented as mean ± standard deviation. NS indicates no tissue samples. The asterisks show the significant difference of gene expression between the young and old groups (P<0.05)

Table 3. *The relative expression of Asian elephant biglycan gene in the wounded samples*

Name	Relative Expression
Momae	0.048±0.009 [a]
Mogradee	0.500±0.154 [b]
Saitong	0.010±0.001 [a]

The data are represented as mean ± standard deviation. [a,b] indicate the significant difference of relative expression among three elephants (P<0.05)

the alteration of expression pattern of *biglycan* by growth factors and pathologic conditions [4].

In this study, the first coding sequence of Asian elephant *biglycan* (1,110 bp) with a deduced amino acid sequence (369 amino acid) and some conserved structures were presented. High expression levels of *biglycan* were found in cartilage and spleen. The expression levels of *biglycan* mRNA that was related to the physiology of wound healing were also revealed. These study provided the preliminary information for further study on developing biomarker for the detection and treatment of disorders related to biglycan in Asian elephant.

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