OSTEOLOGY

Image Processing Technique for Age Estimation in Thai Adults by Histomorphometry of Decalcified Cortical Bone

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ABSTRACT

Objective: **Age estimation is an important step for biological identification. Histomorphometric age estimation in humans is based on observation of microstructural changes in the cortical bone. Several studies have used undecalcified bone sections for histomorphometric age estimation. In Thailand, most hospitals provide facilities and equipment for decalcified methods. Thus, the aim of this study is to develop an age prediction equation using decalcified bone sections stained by picrosirius red in the Thai population.**

Design: **Age at death estimation was analyzed histomorphometrically from the cortical bones of femurs.**

Materials and Methods: **Femoral cortical bone was collected from 71 cadavers (49 males and 22 females) with known age, sex and cause of death. Tissue sections were decalcified and stained with picrosirius red. Histological variables were measured with pixels using an image processing technique and MATLAB program. The histological area was measured in five variables. In addition, this study attempted to develop one new variable for quantifying the measurement of bone collagen.**

Results: **Stepwise multiple regression was employed to develop a predictive model of age estimation. The stepwise selection method in this study utilized the following three histological variables for the equation: the perimeter of Haversian canal, the percentage of lamellar bone area and collagen in the bone stained by picrosirius red. The multiple correlation coefficient and standard error of estimate were 0.906 and 8.26, respectively.**

Conclusion: **The decalcifying procedure and staining method that is routinely employed in histopathology laboratories with image processing technique can assist with age at death estimations in Thailand.**

KEY WORDS

histomorphometry, age estimation, histology, cortical bone, femur

INTRODUCTION

The most common traditional method used to estimate age at death of adult skeletal structures includes evaluation of degenerative changes in the bones[1-4)]. However, it is difficult to reliably estimate the age of individuals older than 50 years[5-8)]. In crime scenes when dealing with incomplete bone or fragmented skeletons it may be necessary to use thin sections for microscopic methods[9)]. Histomorphometry methods quantify changes in cellular activity over time and provide information on bone remodeling[10,11)].

In adults most often age estimation is based on the correlation between increasing age and microstructural changes in cortical bone[10,12-14)]. However, despite the focus on the relationship between age and histomorphometey variables, most analysis is performed by using Image J software[6,15)]. This is the first reported study that has considered the quantitative measurement relationship between age and histomorphometey variables by using an image processing technique in MATLAB.

Bone primarily consists of protein and mineral (mostly hydroxyapatite) content with collagen containing around 33% of the overall proteins and is the key principal component of connective tissues in mammals which makes it the largest single extracellular matrix component[16)]. The most frequently observed collagen is the extracellular matrix type I

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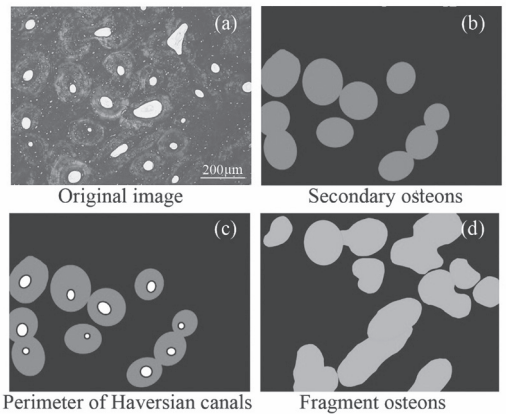

Figure 1. (a) Original image, (b) Secondary osteons with red, (c) Perimeter of Haversian canals with black, and (d) Fragment osteons with green

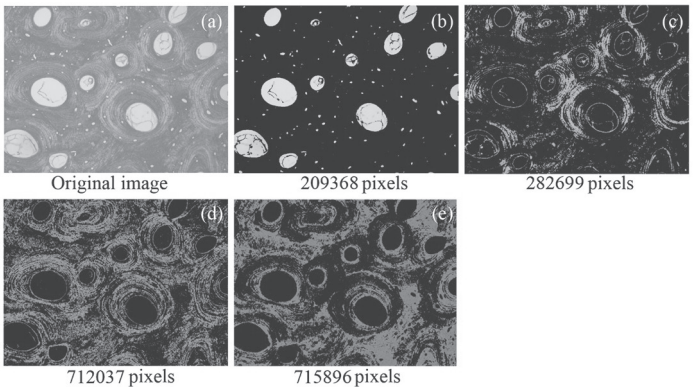

Figure 2. MATLAB programs were divided into 4 sections: (a) Original image stained with picrosirius red, (b) No-red, (c) Light-red, (d) Medium red, and (e) Deep red.

Table 1. Pearson correlation coefficients between histomorphometric variables and age

Variable	On.Ar	On.Fg.Ar	Tt.On.Ar	Pm.H.Ar	Lm.B.Ar	COL.B
Sex-pooled	-0.345 **	0.205	0.720 **	0.856 **	-0.449 **	0.702 **
Male	-0.359*	0.313*	0.709 **	0.868 **	-0.453 **	0.800 **
Female	-0.350	-0.860	0.801 **	0.840 **	-0.495 **	0.467 **

*Statistically significant at the 0.01 level.

**Statistically significant at the 0.05 level.

Table 2. The results of simple linear regression analysis were used to establish predictive formulae for age estimation.

Variable	Formula	r^2	Adjusted r^2	SEE (years)
1.On.Ar	66.977 -0.00001 (On.Ar)	0.119	0.106	18.018
2.On.Fg.Ar	48.506+5.04077 (On.Fg.Ar)	0.042	0.028	18.789
3.Tt.On.Ar	-16.081+0.000025 (Tt.On.Ar)	0.518	0.511	13.331
4.Pm.H.Ar	14.099+0.01438 (Pm.H.Ar)	0.733	0.730	9.911
5.Lm.B.Ar	94.444-0.00002 (Lm.B.Ar)	0.202	0.190	17.152
6.COL.B	-4.840+ 0.00013 (COL.B)	0.493	0.485	13.670

Determination coefficient (r^2), adjusted (r^2), standard error of the estimate (SEE).
On.Ar- Secondary osteon area; On.Fg.Ar- Fragment osteon area; Tt.On.Ar- Total osteon area; Pm.H.Ar- Perimeter of Haversian canal; Lm.B.Ar- Percentage of lamellar bone area;
COL.B- Collagen in bone stained by picrosirius red

collagen[17], which governs the lamellar structure of bone and is arranged in bundles formed by parallel layers[18] within the cortical bones. Extracellular matrix shows changes due to aging as the synthesis of collagen is reduced[19].

Several staining methods that identify collagen in histology include: van Gieson, electron microscope[20], and picrosirius red (PSR)[21]. Traditionally, trichrome stains were used to detect collagen fibers in tissue sections for paraffin-embedded tissue not compatible with plastic-embedded bone[22]. Although previous studies of age estimation by histological methods commonly used preparation of undecalcified bone sections, the preparation of decalcified bone sections and staining included only a few studies[23-25]. In Thailand, the decalcifying and staining procedure of histological method offers low cost and high efficiency and is routinely employed in histopathology laboratories.

The possible benefits of staining connective tissues for observation under polarization microscopy are that collagen bundles reveal a strong birefringence with red color[26,27]. Picroirius red is a well-known method to stain type I collagen fiber in histology[23,24,28]. It is also interesting to examine the relationship between age and collagen histologically prepared by decalcified bone sections stained with picrosirius red.

The aim of this study was to develop an age prediction equation using decalcified bone sections and stained by picrosirius red in Thais. A new variable was developed to study collagen fibers in cortical bone with age and histomorphometric variable analyses using a MATLAB program and image processing technique.

MATERIALS AND METHODS

Bone samples were obtained from 71 Thai cadavers (49 males and 22 females) with known age, sex and cause of death. The individuals ranged in age from 25 to 92 years (mean ± standard deviation: 52.05 ± 19.05 years). The cortical bones were removed and cross-sectioned from the anterior midshaft of the left femurs. Bone samples measured approximately 2.5 x 1.5 centimeters. The cortical bones presenting with mid-diaphysis damage or pathology were excluded. All samples were sent for routine autopsy to the Department of Forensic Medicine and the fresh cadavers were received as body donors from the Department of Anatomy for medical-surgical training at the Cadaveric Surgical Training Center, Faculty of Medicine, Chiang Mai University, Thailand.

Small pieces of bone were washed in water to remove adhering soft tissue. Subsequently, the tissue samples were fixed in 10% formalin for 24 hours, then decalcified using nitric acid solutions at 20% concentrations for five to six days[23] and washed in running tap water for three hours. Following this, the bone tissues were cut into one mm-thick cross section pieces and placed in plastic cassettes. Afterward, the bone tissues were processed in the histology unit and cut into four µm sections.

The tissue sections were deparaffinized in xylene and rehydrated used a graded alcohol series to running tap water for five minutes, followed by staining with Picrosirius Red (PSR, Sigma-Aldrich, Dorset, UK) for one hour[29] and washed in acidified water for one minute. The sections were dehydrated in graded ethanol series and mounted by Permount.

The bone sections were observed per two fields (10 x objective) under a compound light microscope Olympus BX53. All measurements were done in digital JPG files (image size: 4800 x 3600 pixels) to determine the number of pixels in an image, then each pixel was used to calculate the area of variables using a MATLAB program version 2016 and image processing technique as shown in (Figures 1 and 2).

Six variables in cross-section were observed. The histological variables one through five selected for this study were identified and adapted as described below[8,23,30,31] with the variables expressed in square micrometers (µm^2) (Figure 1).

1. Secondary osteon area (On.Ar): Average surface area of second-

ary osteons, when 80% of the reversal line was contained within the secondary osteons.
2. Fragment osteon area (On.Fg.Ar): Average surface area of fragmented osteons. When an osteon was a partially remodeled osteon with an incomplete reversal line.
3. Total osteon area (Tt.On.Ar): The sum of On.Ar and On.Fg.Ar.
4. Perimeter of Haversian canal (Pm.H.Ar): Mean length of the outer edge of the Haversian canal.
5. Percentage of lamellar bone area (Lm.B.Ar): Percentage of lamellar bone surface per unit area.
6. Collagen in bone stained by picrosirius red (COL.B): Average red color in bone per unit area (Figure 6.

Image Processing Technique: Method was Developed in Two Ways: Analysis Histomorphometry (variables in 1-5)

The variables from the original image were separated into three sections of image segmentation. The unit area of secondary osteons and fragment osteons was then divided in order for the program to draw and replace the position of the secondary osteons with red, the fragment osteons with green, and perimeter of Haversian canal with black, as shown in (Fig. 1). Moreover, the program was used to retrieve any needed variables from the original image, and then replace the background of the image with black, then transformed the image to gray, followed by converting the gray image to a binary image. The final image was used to sum the number of pixels and count the white pixels in all variables, then the number of pixels were used to calculate the area with age by an image processing technique.

Collagen in Bone Stained by Picrosirius Red (variable in 6)

The variables from the original image were produced by the MATLAB program using image processing and divided into four sections: no-red, light-red, medium red and deep red (Fig. 2). Each variable brought out the color from the original image using an image estimation method, replaced the blank spaces with black, and then transformed the image to gray, converting the gray image to a binary image. The final image was used to sum the number of pixels and count the white pixels in a total of four sections. Then, the number of pixels were used to calculate the area with age by an image processing technique.

RESULTS

The relationship between age and the pixel density of histological variables was evaluated using Pearson's correlation with the results presented in Table 1. The relationship was positive for four variables in the sex-pooled, abbreviations for histomorphometric: (On.Fg.Ar), (Tt. On.Ar), (Pm.H.A), and (COL.B). Meanwhile, (Pm.H.A) showed the highest correlation coefficient with age, which was (r = 0.856), as shown in Table 1.

Table 2 shows that the determination coefficients as presented indicated that Pm.H.Ar (r^2 = 0.733) is the variable most closely correlated with age. Simple linear regression was used to establish a predictive formula for each variable for age estimation showed in six variables. This study found one variable: Pm.H.Ar with the lowest standard error of estimate of 9.91 years.

The stepwise multiple regression equations shows the most accurate predictive model and formula established in this study, which found that three variables that best predicted age consisted of; Pm.H.Ar, Lm.B.Ar and COL.B. The equations predictive for age estimation were: -28.199 + 0.0138 (Pm.H.Ar) + 0.00005 (COL.B) + 9.312 (Lm.B.Ar). The multiple correlation coefficient and standard error of estimate were 0.906 and 8.26, respectively.

DISCUSSION

Age at death estimation using histomorphometric methods has been widely used across the globe. In 1965, Kerley was the first author to study histomorphometry of cortical bone. The most typical variables used methods such as counting the number of osteons, number of osteon fragments, which could be used to estimate the age at death of adult skeletons[8,13,32,33]. However, histomorphometric methods have never been studied in a Thai research population. Thus, in the present study, we focused on measuring the relationships between age and histological area variables.

In the present study, the correlation coefficients between average secondary osteon areas tended to decrease with age, and it was similar to the studies of Goliath, Yoshino and Watanabe[6,8,34]. Additionally, the number of secondary osteons increasing with age have been reported by Yoshino, Kerley, Ericksen, and Keough[8,13,32,33]. Therefore, these age-associated increases occur in the total number of osteons per unit area[6,8], while lamellar bone is supplanted by osteons, and the bigger osteons of early life are substituted by smaller osteons of maturity and old age[35]. Consequently, the correlation coefficients between the percentages of lamellar area that tended to decrease with age increased[23], in this present study the lamellar area with age was (r = -0.449), therefore, our present study confirms that the lamellar area tended to decrease per unit with age.

To equate our present data with that of previous literature would be problematic due to differences in sample size, type of bones, methods, variables and population groups. In our study, the reason for selecting decalcified methods is that most hospitals in Thailand have the facilities and equipment for performing decalcified methods. These decalcified methods and stained bone sections are routine work in pathology labs, and these methods have low costs and high efficiency. In addition to the ability to selectively stain collagen, including sites of examination having type I collagen, picrosirius red stain is ideal for utilization during studies with polarization microscopy[36].

Histomorphometry analyses were performed using Image J software which requires no cost to download from the internet, while commercially available software is costly[37]. This is one of the first studies to use computer assisted images based on the quantitative measurements of image segments, which is a process of dividing the digital image into multiple sections using a MATLAB program. In addition, the advantages of this image processing technique have been used in histopathology images for segmentation and classification structure in organs, measuring cell shapes, and disease classification if any is present in the sample[38]. Furthermore, equations derived from this study can be developed into application software for ease of use and saving time in image analysis. Further studies can use these neural networks together in order to analyze the data from image processing techniques for more accurate results.

In this study we found that collagen in males and females had a correlation with age, (r = 0.800 and r = 0.467) respectively. An increase in fragility within the bone matrix is one result of increased metabolism and explains the observed post-translational modifications of collagen. Consequently, bone metabolism has an important role in the pathogenesis of osteoporosis, which is of particular concern for middle-aged women during menopause because of the increased activity rate of the basic multicellular units for these individuals. In addition, there are several hormones that directly and indirectly affect bone remodeling in adults such as the parathyroid hormone (PTH) and glucocorticoids[39]. Many factors, such as lifestyle, metabolism, diet, disease states, and growth trajectories[40] are also known to affect the organization of collagen fibers within the cortical bone.

CONCLUSION

In this study, our results found that (Pm.H.Ar), (Lm.B.Ar), and a new variable (COL.B) were used to develop age at death estimation in the Thai population. Furthermore, the decalcifying procedure and picrosirius red staining method, which are routine techniques employed in histopathology laboratories, can be applied to incomplete skeletons and estimation of age at death in the Thai adult population. The application of software with an image processing technique is a potential tool in forensic cases.

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REFERENCES

1) İŞcan MY, Loth SR, Wright RK. Age estimation from the rib by phase analysis: white males. J Forensic Sci 1984; 29(4): 1094-104.
2) Buckberry JL, Chamberlain AT. Age estimation from the auricular surface of the ilium: a revised method. Am J Phys Anthropol 2002; 119(3): 231-9.
3) Todd TW, Lyon DW. Endocranial suture closure its progress and age relationship. Am J Phys Anthropol 1924; 7: 325-84.
4) Passalacqua NV. Forensic age-at-death estimation from the human sacrum. J Forensic Sci 2009; 54(2): 255-62.
5) Martrille L, Ubelaker DH, Cattaneo C, Seguret F, Tremblay M, Baccino E. Comparison of four skeletal methods for the estimation of age at death on white and black adults. J Forensic Sci 2007; 52(2): 302-7.
6) Goliath JR, Stewart MC, Stout SD. Variation in osteon histomorphometrics and their impact on age-at death estimation in older individuals. Forensic Sci Int 2016; 262: 282.e1-6.
7) Falys CG, Schutkowski H, Weston DA. Auricular surface aging: worse than expected? A test of the revised method on a documented historic skeletal assemblage. Am J Phys Anthropol 2006; 130(4): 508-13.
8) Yoshino M, Imaizumi K, Miyasaka S, Seta S. Histological estimation of age at death using microradiographs of humeral compact bone. Forensic Sci Int 1994; 64: 191-8.
9) Nganvongpanit K, Phatsara M, Settakorn J, Mahakkanukrauh P. Differences in compact bone tissue microscopic structure between adult humans (Homo sapiens) and Assam macaques (Macaca assamensis). Forensic Sci Int 2015; 254: 243.e1-5.
10) Kulak CA, Dempster DW. Bone histomorphometry: a concise review for endocrinologists and clinicians. Arq Bras Endocrinol Metab 2010; 54(2): 87-98.
11) Crowder C, Stout S, eds. Bone Histology: An Anthropological Perspective. CRC Press, 2011.
12) Maat GJ, Maes A, Aarents MJ, Nagelkerke NJ. Histological age prediction from the femur in a contemporary Dutch sample: The decrease of nonremodeled bone in the anterior cortex. J Forensic Sci 2006; 51(2): 230-37.
13) Kerley ER. The microscopic determination of age in human bone. Am J Phys Anthropol 1965; 23(2): 149-63.
14) Britz HM, Thomas CD, Clement JG, Cooper DM. The relation of femoral osteon geometry to age, sex, height and weight. Bone 2009; 45(1): 77-83.
15) Sobol J, Ptaszyńska-Sarosiek I, Charuta A, Oklota-Horba M, Żaba C, Niemcunowicz-Janica A.Estimation of age at death: examination of variation in cortical bone histology within the human clavicle. Folia Morphol (Warsz) 2015; 74(3): 378-88.
16) Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem 2009; 78: 929-58.
17) Patino MG, Neiders ME, Andreana S, Noble B, Cohen RE. Collagen: An Overview. Implant Dent 2002; 11(3): 280-5.
18) Firestein GS, Budd RC, Gabriel SE, Mcinnes IB, O'dell JR. Kelley's Textbook of Rheumatology. 10th edition. Elsevier, 2017.
19) Panwar P, Lamour G, Mackenzie NC, Yang H, Ko F, Li H, *et al*. Changes in structural-mechanical properties and degradability of collagen during aging-associated modifications.
J Biol Chem 2015; 290(38): 23291-306.
20) Hulmes DJ, Jesior JC, Miller A, Berthet-Colominas C, Wolff C. Electron microscopy shows periodic structure in collagen fibril cross sections. Proc Natl Acad Sci U S A 1981; 78(6): 3567-71.
21) Puchtler H, Waldrop FS, Valentine LS. Polarization microscopic studies of connective tissue stained with Picro-sirius red FBA. Beitr Pathol 1973; 150(2): 174-87.
22) Villanueva AR, Mehr LA. Modifications of the Goldner and Gomori one-step trichrome stains for plastic-embedded thin sections of bone. Am J Med Technol 1977; 43(6): 536-8.
23) Martrille L, Irinopoulou T, Bruneval P, Baccino E, Fornes P. Age at death estimation in adults by computer-assisted histomorphometry of decalcified femur cortex. J Forensic Sci 2009; 54(6): 1231-7.
24) Cannet C, Baraybar JP, Kolopp M, Meyer P, Ludes B. Histomorphometric estimation of age in paraffin-embedded ribs: a feasibility study. Int J Legal Med 2011; 25(4): 493-502.
25) Singh IJ, Gunberg DL. Estimation of age at death in human males from quantitative histology of bone fragments. Am J Phys Anthropol 1970; 33(3): 373-81.
26) Piérard GE. Sirius Red Polarization method is useful to visualize the organization of connective tissues but not the molecular composition of their fibrous polymers. Matrix 1989; 9(1): 68-71.
27) Stephenson B. A modified Picro-Sirius Red (PSR) staining procedure with polarization microscopy for identifying collagen in archaeological residues. J Archaeol Sci 2015; 61: 235-43.
28) Vogel B, Siebert H, Hofmann U, Frantz S. Determination of collagen content within picrosirius red stained paraffin-embedded tissue sections using fluorescence microscopy. MethodsX 2015; 21(2): 124-34.
29) Kiernan AJ. Sirius Red for collagen staining protocol. Department of Anatomy & Cell Biology, The University of Western Ontario, London, Canada N6A 5C1.
30) Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, *et al*. Bone histomorphometry: standardization of nomenclature, symbols and units. J Bone Miner Res 1987; 2(6): 595-610.
31) Pfeiffer S. Variability in osteon size in recent human populations. Am J Phys Anthropol 1998; 106(2): 219-27.
32) Ericksen MF. Histologic estimation of age at death using the anterior cortex of the femur. Am J Phys Anthropol 1991; 84(2): 171-9.
33) Keough N, L'Abb EN, Steyn M. The evaluation of age-related histomorphometric variables in a cadaver sample of lower socioeconomic status: implications for estimating age at death. Forensic Sci Int 2009; 191(1-3): 114.e1-6.
34) Watanabe Y, Konishi M, Shimada M, Ohara H, Iwamoto S. Estimation of age from the femur of Japanese cadavers. Forensic Sci Int 1998; 98(1-2): 55-65.
35) Donald JO. Aging effects on osteon remodeling. Calcif Tissue Re 1975; 18(1): 27-36.
36) Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. J Histochem 1979; 11(4): 447-55.
37) Egan KP, Brennan TA, Pignolo RJ. Bone histomorphometry using free and commonly available software. Histopathology 2012; 61(6): 1168-73.
38) Belsare AD. Histopathological image analysis using image processing techniques: An overview. signal & image processing. An International Journal 2012; 3: 23-36.
39) Martin RB, Burr DB, Sharkey NA, des. Skeletal tissue mechanics. New York Inc: Springer, 1998.
40) Goldman HM, Bromage TG, Thomas CD, Clement JG. Preferred collagen fiber orientation in the human mid-shaft femur. Anat Rec A Discov Mol Cell Evol Biol 2003; 272(1): 434-45.