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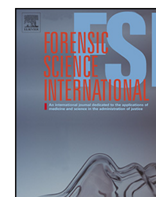

Usefulness of protein analysis for detecting pathologies in bone remains

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ABSTRACT

Forensic pathology often uses osteobiography, which involves biological profiles based on a determination of the age, sex, constitution, pathological states and other anomalies (paleopathology) of subjects for identification purposes. In this paper, proteins were analysed in bone remains. A total of 45 long bones from 45 different cadavers (29 males, 16 females) with a mean age of 66.31 years (S.D. = 19.48, range 20–97) were used to search for pathological biomarkers which are closely related to several diseases. The bones were removed from the cement niches of a cemetery in Murcia (south-eastern Spain), where they had lain for between 18 and 45 years (mean time 25.84 years, S.D. = 8.91). After a specific extraction using Tris-Urea buffer, were measured using HPLC/MS/MS. Our results show that proteins resulting from tumoral diseases and bacterial and viral pathogens can be detected and identified in the skeletal remains, making them useful pathological biomarkers for constructing biological profiles.

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1. Introduction

The main aim of forensic pathology is the study of human remains, particularly skeletal remains, and their identification. In this last case, biological profiles are constructed based on the determination of age, sex, race, stature, pathologies and other anomalies (paleopathology), creating, in other words, an osteobiography that may be of use in the case of unidentified human remains [1].

Different pathologies may produce alterations in the structure and composition of bones, so that any information in this respect may be important for confirming the presence of such diseases. Moreover, changes related with traumatisms, nutritional deficiencies or other pathologies (bacteria, viruses, tumors, etc.) may persist in bones [2,3].

Several proteins have been mentioned as possible markers of pathologies in biological samples of patients, including Procollagen Type 1 Nitrogenous Propeptides (P1NP), osteocalcin (OC) and β isomerized type I collagen C-telopeptides (β CTX), all biochemical markers of bone turnover for hyperthyroidism [4], and bone markers such as OC and C-terminal cross-laps (CTX) for *Human immunodeficiency virus* (HIV) [5], tartrate-resistant acid phosphatase (TRAP) 5b as serum marker of bone metastases in human breast cancer [6] and P1NP and CTx for prostate cancer [7]; and Fms-like tyrosine kinase 3 (flt3) for acute amyloid leukaemia [8], among others. Moreover studies have been made of the proteins of infectious agents, such as *Moraxella catarrhalis* and *Giardia intestinalis ATCC 50581* in the heces of children [9,10] or of *Photorhabdus asymbiotica* in the blood and tissues of affected patients [11].

Postmortem disease markers for the Hepatitis C virus and for HIV were studied by Cattaneo et al. [12] through the detection of antigens, while recent studies by Bona et al. [13] point to the possibility of detecting tumoral markers. For example, annexin A10 protein, BCL-2-like protein, calgizzarin, HSP beta-6, Rho GAP-activating protein 7, transferrin and vimentin could indicate the presence of osteosarcoma in bone tissues in tumorous skeletal remains of up to 2000 years old.

Cattaneo [1] suggested that a body becomes skeletonized after about five years and that histological changes of the compacted bone begin at around the same time. The material selected for study in our case was the compacted bone tissue of long bones, which is the most resistant material postmortem, the femur in particular offering a good degree of conservation for molecular analysis and could be individualized [14].

The aim, therefore, of this study was to analyse the possibility of characterising the proteins present in the bone matrix, using

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analytical techniques that provide information on the pathological antecedents of the subject in real time.

2. Materials and methods

2.1. Bone samples

A total of 45 long bones (33 femurs and 12 tibias) from 45 different cadavers (29 males, 16 females) with a mean age of 66.31 years (S.D. = 19.48, range 20–97) were used (Table 1). The bones were removed from cement niches of a cemetery in Murcia, a city in the southeast of Spain, an area with a very low rainfall index. Winters are mild with temperatures that range between 5 and 19 °C, while summers are hot with temperatures that range between 22 and 40 °C. The cemetery is situated in a dry area and on land which is rich in lime and gypsum. The bones had lain in the niches for documented times of between 18 and 45 years (mean time 25.84 years, S.D. = 8.91). The date of the death was obtained from the cemetery registry office and was later checked according to civil registry documents. The cadavers, which were entirely skeletal, were buried in such a way that the bone remains did not come into contact with soil. As a result, the processes that usually occur after burial, including chemical soil factors that induce transformations of the bone mineral matrix were minimal.

Taking into account that the bones were identified and authenticated using perfectly reliable data, we did not use any bone reference standards, although internal standards were used throughout in order to check the reliability and precision of the analyses.

For the 45 cadavers used in the study, 29 (64.4%) clinical histories were available, of which 14 (48.3%) reflect the medical records of the pathologies related with the identified proteins. In the remaining cases, insufficient information was available to corroborate the pathology described with the proteins identified in this study The main pathologies that were included in the clinical histories were HIV ($n = 8$), cancer ($n = 6$), leukaemia ($n = 2$), anaemia ($n = 3$) and amyotrophic lateral sclerosis 2 ($n = 2$), although the presence of bacteria and other viruses was not included, and there were pathologies at the time of death that were not identified (herpes, parvovirus, etc.).

Table 1
Number of the sample, age of the subjects and postmortem interval.

Number of the sample	Age of the subjects	Postmortem interval
1[**]	48	18
2	36	18
3[*]	59	18
4[**]	74	18
5[*]	20	18
6[**]	93	18
7[*]	58	18
8[**]	92	18
9[*]	69	18
10[*]	64	18
11[**]	67	19
12[*]	71	19
13[*]	33	19
14	84	20
15	47	20
16	21	20
17[*]	58	20
18[*]	76	21
19[**]	67	21
20	79	21
21	85	21
22[**]	78	21
23	39	21
24	42	22
25	84	23
26[*]	75	23
27[**]	67	23
28[**]	33	24
29	97	24
30[*]	78	24
31	90	24
32	69	30
33[**]	76	31
34	82	31
35[**]	53	34
36	77	35
37	91	39
38[**]	82	40
39[*]	48	40
40[**]	75	40
41[*]	57	41
42	86	42
43[*]	58	42
44[*]	78	43
45[**]	68	45

[*] Subjects for whom the medical record is available but which does not provide information related with infectious pathology markers.
[**] Subjects whose medical record reflect pathologies which coincide with the identified proteins. Note that in the period covered by the medical record the infectious agents were neither detected nor characterized. Common place infections were not normally included in the records.

2.2. Extraction of proteins

Sample preparation, separation and mass spectrometric analysis are of vital importance for the quality of the results. Here, we used the method described by Jiang et al. [15] and Schweitzer [16] with some modifications for non-collagen protein (NCP) extraction. First, 350 mg of crude bone powder were washed to remove contaminants with phosphate buffer saline 1 M (pH 7.4) for 48 h at 24 °C. Before extraction, the samples were centrifuged for 10 min at 4000 rpm in a benchtop microfuge. The supernatant (acid-insoluble fraction) was collected and the remaining pellets were stored at 4 °C. Next, the NCPs were extracted for 48 h at 4 °C in a buffer containing 100 mM Tris and 6 M Urea at pH 7.4.

2.3. Mass spectrometry

A 40 μl volume of each sample was analysed by HPLC/MS/MS, using Agilent® 1100 Series (Agilent Technologies, Santa Clara, CA, USA) apparatus coupled to a mass spectrometer (120 k resolution, full scan, positive mode, normal mass range 350–1500). Peptides in the sample were separated on a Waters Xbridge BEH300 C18 (150 mm × 1 mm i.d., 5 μm), using a gradient from 100% A (0.1% formic acid in water) and 0% B (0.1% formic acid in acetonitrile) to 80% B in 180 min at a flow rate of 10 μl min^{-1}. Then, the peptides were automatically selected for fragmentation by data-dependent analysis; AutoMS (Agilent Ion Trap XCT Plus mass spectrometer), product ion scans, rapid scan rate, Centroid data; scan event: 1000 count minimum signal threshold, top 3) were acquired per cycle, dynamic exclusion was employed, and 1 repeat scan (i.e. two MS/MS scans total) was acquired in a 30 s repeat duration with the precursor being excluded for the subsequent 30 s (activation: collision-induced dissociation (CID), 2+ default charge state, 2 m/z isolation width, 35 eV normalized collision energy, 0.25 activation Q, 10.0 ms activation time).

2.4. Data handling

Peptide masses obtained via HPLC/MS/MS were compared with the National Center for Biotechnology Information (NCBInr) database for matching primary protein sequences using the Data Analysis for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany) and Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, CA, USA).

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