Mass Spectrometric Identification of Ancient Proteins as Potential Molecular Biomarkers for a 2000-Year-Old Osteogenic Sarcoma

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Abstract

Osteosarcoma is the most common primary malignant tumor of bone usually occurring in young adolescent and children. This disease has a poor prognosis, because of the metastases in the period of tumor progression, which are usually developed previous to the clinical diagnosis. In this paper, a 2000-year-old ancient bone remain with osteogenic sarcoma was analyzed searching for tumor biomarkers which are closely related to this disease. After a specific extraction SDS-PAGE gel electrophoresis followed by tryptic digestion was performed. After the digestion the samples were measured using MALDI TOF/TOF MS. Healthy bone samples from same archaeological site were used as control samples. Our results show that in the pathological skeletal remain several well known tumor biomarkers are detected such as annexin A10, BCL-2-like protein, calgizzarin, rho GTPase-activating protein 7, HSP beta-6 protein, transferrin and vimentin compared to the control samples. The identified protein biomarkers can be useful in the discovery of malignant bone lesions such as osteosarcoma in the very early stage of the disease from paleoanthropological remains.

Introduction

Osteosarcoma is the most common primary bone tumor characterized by the production of ostoid matrix from malignant cells. It typically occurs in the long bones of the near metaphyseal growth plates of children and young adolescents [1]. The earliest known case affected a male Celt (ca. 800–600 BC) from Switzerland with a possible osteosarcoma or chondrosarcoma [2]. A possible osteosarcoma of the pelvis has been noted in a young individual from Ancient Egypt, dating to about 250 AD [3], and a well-documented case of osteosarcoma, with the typical radiographic “sunburst” pattern, has been reported in the femur of a native Peruvian dating to 800 BP [4]. Additional cases of osteosarcoma have been observed in a young female femur from the prehistoric population of Oahu in Hawaii [5], and in a zygomatic bone from the French Middle Ages [6] in a case of 17th century mandible from West Virginia [7]. Possible osteosarcomas have been detected in a young male from the Saxon necropolis of Standlake, England [8] and in medieval skulls from the Czech Republic [9] and France [10]. Probable cranial hemangiosarcoma has been documented in an elderly female from Italy, 3rd Century BC [11] and in a humerus from Peru, 12–14th Centuries AD [4] and a possible Ewing’s sarcoma in a juvenile skull from Bronze Age of Tartaren, Spain [12]. Only a few cases of neoplasms have been documented in Central and South American mummies, for example a rhabdomyosarcoma (4–7th Centuries AD) in 2 children from Chile [13].

Diagnosis of cancer as well as osteogenic sarcomas from ancient human skeletal remains is not an easy task by using classic morphological methods. Therefore a biomolecular approach to diagnosis in addition to osteological examination can be beneficial [14]. Recently, proteomic profiling of human tumors has provided a better understanding of the molecular pathogenesis of neoplastic diseases and has identified novel biomarkers for early diagnosis. SELDI-TOF-MS (Surface Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry) and protein microarray high throughput analysis enable to detect biomarkers from the serum samples of osteosarcoma patients [15]. Based on the MALDI-TOF analyses of clinical benign and osteosarcomas several biomarkers are up- or down-regulated [16]. Also MALDI-TOF analysis of human osteosarcoma MG-63 cells showed the alterations of some genes and proteins [17].

Additionally, mass spectrometry (MS) based proteomic studies of paleopathological remains have made sequence information...
At this site we uncovered 33 graves from the late Roman period, arranged in four rows, very close to each other. Grave 186 (indicated with a red arrow) is located in the south-eastern section of the site at 270–90°. The upper perimeter dimensions of the grave were 218 by 126 cm, the base 185 by 120 cm, with a depth of 48 cm.

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Figure 2. The paleoanthropological sample chosen for mass spectrometric analysis. A) and B) Anterior and posterior views of the analyzed right humerus with osteogenic sarcoma. C) X-ray radiograph of the measured human remain. The red ellipse shows the location of the sampling.

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available from subpicomolar quantities of fragmented proteins and peptides [18–26].

In this study, ancient proteins such as malignant bone tumor related molecular biomarkers were successfully extracted and detected from archaeological human skeletal remains by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI TOF/TOF MS) for the first time. Our proteomic results can enhance the diagnosis of osteogenic tumors in ancient human skeletal remains.

Materials and Methods

Ethics Statement

**Specimen numbers.** Savaria, Szent Marton str. 53/grave 186 (osteosarcoma) and grave 199 (healthy control).

**Repository information.** Savaria Museum, Archaeological Collection, Kisfaludy str. 9, H-9700 Szombathely, Hungary. Department of Anthropology, University of Szeged, Kozépfalasz 52, H-6726, Szeged, Hungary.

All necessary permits were obtained for the described study, which complied with all relevant regulations (National Office of Cultural Heritage permission number: 3495/2001).

Archaeological Bone Sample

The fragmented skeleton of a 25–35-year-old female has been excavated at the Late Roman archaeological site of Szombathely (Savaria) - Szent Marton street 53, Hungary (municipal location code 6583/1) grave 186 (Figure 1). The analyzed bone sample was collected from the cortical region of the right humerus (Figure 2). The anthropological and paleopathological investigations were carried out based on Knussmann [27] and Jozsa [28]. A humerus from a non-cancerous remain from this cemetery (grave 199, adult female) was used for the investigations as a control sample. For further validation we used our previous proteomic results of some non-pathological and *M. tuberculosis* infected bone samples (Table 1 and 2).

Extraction of Ancient Proteins

The sample preparation, separation and mass spectrometric analysis is of a vital importance for the quality of the results. In our previous study we developed an optimized workflow for proteomic analysis of ancient proteins [19]. Here, we used this method with some modifications. Briefly, the bone fragments were washed to remove contaminants with phosphate buffer saline (PBS) and distilled water. Bone powder was ground by hand with an agate mortar, the particle size was \(0.2\) mm. Next, 100 mg of crude bone powder was decalcified with 1.00 ml of 0.5 M EDTA (pH = 8.0), the pellet was resuspended with 100 \(\mu\)l of 6 M guanidine-HCl in 0.1 M Tris (pH = 7.5) at room temperature.

### Table 1. The analyzed healthy control bone samples.

| Location          | Grave | Age | Sex   | Period            |
|-------------------|-------|-----|-------|-------------------|
| Mohacs            | -     | Adult | Male | Recent (forensic) |
| Bataszek          | -     | Adult | Male | Recent (forensic) |
| Bataszek          | -     | Adult | Female | Recent (forensic) |
| Mohacs            | -     | Adult | Female | Recent (forensic) |
| Pecs              | -     | Adult | Female | Recent (forensic) |
| Hodmezovasarhely-Gorza 16 | Senium | Female | Neolithic |
| Mezökövesdi Patakrajaro | 4/A | Adultus | Male | Chalcolithic |
| Kiskundorozsma    | 15    | Adultus | Female | Bronze Age |
| Algyo Baraktabor  | 4     | Maturus | Male | Scythian |
| Szegvar Oromdulo  | 918   | Adultus | Female | Sarmatian |
| Kishomok          | 89    | Maturus | Female | Gepids |
| Szegvar Oromdulo  | 740   | Adultus | Female | Early Avar |
| Székeskutas Kapholndulo | 30 | Adultus | Female | Late Avar |
| Kiskundorozsma    | 100   | Senium | Male | Hungarian Conquest |
| Esztergalyhorvath | 284   | Adultus | Female | Hungarian Conquest |
| Kecskemet Torekfaj | 65    | Maturus | Female | Arpad Age, X-XIth AD |
| Szegvar Oromdulo  | 617   | Maturus | Female | Arpad Age, XIth AD |
| Derekgégyhaza Ilyolas | 13 | Senium | Male | XI-XIIth AD |
| Csengele Bogarth  | 57    | Adultus | Male | XIIIth AD |
| Kecskemet Ferences church | 350 | Adultus | Female | XIV-XVIIth AD |

| Location          | Grave | Age | Sex | Period |
|-------------------|-------|-----|-----|--------|
| Sukosd-Sagod      | 19    | Adult | Female | VII-VIIIth AD |
| Sukosd-Sagod      | 19    | Adult | Female | VII-VIIIth AD |
| Bacsalmas-Homokbanya | 39 | Maturus | Male | XVIIth AD |
| Bacsalmas-Homokbanya | 39 | Maturus | Male | XVIIth AD |
| Belmega-Csomoki hill | 65 | Maturus | Female | VIIIth AD |
| Belmega-Csomoki hill | 65 | Maturus | Female | VIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Felgyo   | 205   | Adult | Female | VIIIth AD |
| Csöngrad-Felgyo   | 205   | Adult | Female | VIIIth AD |

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Table 2. The measured pathological control (*Mycobacterium tuberculosis* infected) archaeological bone samples.

| Location          | Grave | Age | Sex | Period |
|-------------------|-------|-----|-----|--------|
| Sukosd-Sagod      | 19    | Adult | Female | VII-VIIIth AD |
| Sukosd-Sagod      | 19    | Adult | Female | VII-VIIIth AD |
| Bacsalmas-Homokbanya | 39 | Maturus | Male | XVIIth AD |
| Bacsalmas-Homokbanya | 39 | Maturus | Male | XVIIth AD |
| Belmega-Csomoki hill | 65 | Maturus | Female | VIIIth AD |
| Belmega-Csomoki hill | 65 | Maturus | Female | VIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Elles    | 183   | Maturus | Male | XI-XIIIth AD |
| Csöngrad-Felgyo   | 205   | Adult | Female | VIIIth AD |
| Csöngrad-Felgyo   | 205   | Adult | Female | VIIIth AD |

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Figure 3. Characteristic 1D SDS PAGE electrophoretogram of healthy control and the pathological bone samples. Lanes 1 and 2 are healthy control samples from Hodmezovasarhely-Gorza and Szegvar-Oromdulo, lanes 3–6 are osteosarcoma samples. Arrows show the spots of Annexin A10 (37.3 kDa) and Vimentin (26.8 kDa). The parameters of the separation are mentioned in the text.

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Table 3. The identified up-regulated proteins from 2000-year-old osteogenic sarcoma.

| Accession     | Name                                                      | MW [kDa] | Peptides | SC [%] |
|---------------|-----------------------------------------------------------|----------|----------|--------|
| AK1A1_HUMAN   | Alcohol dehydrogenase (NADP+)                            | 36.5     | 5        | 14.2   |
| gi|225939       | aldehyde reductase                                        | 36.3     | 5        | 14.2   |
| gi|48762937      | annexin A10                                               | 37.3     | 8        | 27.2   |
| gi|62087552      | arginine/serine-rich splicing factor 6 variant            | 31.8     | 6        | 27.6   |
| ARISB_HUMAN   | AT-rich interactive domain-containing protein 5B          | 132.2    | 11       | 13.0   |
| gi|33878074      | BAT2 protein                                              | 17.1     | 5        | 29.9   |
| gi|49456879      | BCL2A1                                                    | 20.3     | 7        | 44.0   |
| VMDL3_HUMAN   | Bestrophin-4                                              | 76.1     | 7        | 14.5   |
| gi|882391        | bone morphogenic protein type II receptor                 | 59.9     | 6        | 13.2   |
| S10AB_HUMAN   | Calgizzarin (S100 calcium-binding protein A11)            | 11.7     | 4        | 43.8   |
| CANT_HUMAN    | Calpain-7                                                 | 92.6     | 7        | 12.3   |
| K1C10_HUMAN   | Cytokeratin 10                                            | 59.5     | 14       | 23.1   |
| gi|33188433      | deleted in liver cancer 1 isoform 1                      | 170.5    | 13       | 7.9    |
| gi|28704113      | DHX8 protein                                              | 138.7    | 13       | 13.8   |
| G59435        | DLC-1                                                     | 122.7    | 10       | 10.9   |
| DNL3_HUMAN    | DNA ligase III                                            | 102.6    | 10       | 16.3   |
| TDT_HUMAN     | DNA nucleotidyltransferase                                | 58.4     | 9        | 22.2   |
| MP2K6_HUMAN   | Dual specificity mitogen-activated protein kinase kinase 6| 37.5     | 7        | 23.4   |
| DTNA_HUMAN    | Dystrobrevin alpha has been deleted                      | 83.9     | 6        | 10.5   |
| gi|15010856      | galectin-12 isoform d                                     | 30.0     | 5        | 30.5   |
| GCC2_HUMAN    | GRIP and coiled-coil domain-containing protein 2          | 184.5    | 18       | 12.3   |
| gi|40555827      | heat shock factor protein 2 isoform c                     | 27.0     | 7        | 23.9   |
| HSPB6_HUMAN   | Heat-shock protein beta-6                                 | 17.1     | 5        | 35.0   |
| CAC10772      | Immunoglobulin heavy chain variable region               | 12.4     | 5        | 65.5   |
| gi|17318569      | keratin 1                                                 | 66.0     | 15       | 25.8   |
| QBN175_HUMAN  | Keratin 10                                                | 58.8     | 14       | 23.5   |
| gi|31559819      | keratin 25C                                               | 49.8     | 8        | 17.2   |
| gi|47132620      | keratin 2a                                                | 65.4     | 9        | 16.4   |
| CAAB2315      | keratin 9                                                 | 62.1     | 11       | 16.9   |
| A44861        | keratin, 67K type II epidermal                           | 65.8     | 9        | 16.3   |
| K1C9_HUMAN    | Keratin, type I cytoskeletal 9                            | 61.9     | 11       | 20.7   |
| AAP97338      | Methyl-CpG-binding domain protein 4                      | 60.9     | 13       | 16.3   |
| NEBL_HUMAN    | Nebulette                                                 | 116.4    | 14       | 16.7   |
| NRAP_HUMAN    | Nebulin-related-anchoring protein                         | 197.0    | 24       | 14.9   |
| gi|4506335       | parvalbumin                                               | 12.1     | 8        | 56.4   |
| PRVA_HUMAN    | Parvalbumin alpha                                         | 11.9     | 8        | 56.9   |
| gi|39653232      | PHD finger protein 20-like 1 isoform 1                    | 47.7     | 7        | 15.8   |
| gi|39653231      | PHD finger protein 20-like 1 isoform 3                    | 16.6     | 6        | 42.0   |
| gi|31873386      | phospholipase C                                           | 39.7     | 7        | 30.7   |
| gi|346323        | phosphoprotein phosphatase (EC 3.1.3.16) X catalytic chain| 35.1     | 6        | 26.1   |
| PDIP2_HUMAN   | Polymerase delta-interacting protein 2                    | 42.0     | 7        | 22.0   |
| CAF00150      | Proteasome subunit beta type 3                            | 16.6     | 5        | 34.9   |
| gi|565647        | proteasome subunit HsC10-II                              | 22.9     | 5        | 25.4   |
| PARK7_HUMAN   | Protein DJ-1 (Oncogene DJ1)                              | 19.9     | 5        | 32.3   |
| gi|55859594      | PTAR1 protein                                             | 32.4     | 7        | 30.1   |
| gi|4960030       | Rab GDP dissociation inhibitor beta                        | 41.0     | 6        | 22.0   |
| gi|39841018      | RAB GTPase activating protein 1-like                      | 92.5     | 8        | 10.3   |
| gi|537327        | receptor tyrosine kinase                                  | 18.5     | 6        | 30.0   |
| gi|2665850       | rheumatoid factor RF-ET7                                  | 10.9     | 6        | 74.5   |
The extraction of the proteins was carried out by continuous shaking at 4°C for 8 hours with the presence of protease inhibitor cocktail (Sigma Aldrich Kft., Budapest, Hungary). The protein extract was purified by using C₁₈ solid phase extraction (SPE) cartridge. For this purification step a homemade octadecylsilane modified silica-based stationary phase was used with average particle size of 5 μm and pore size of 120 Å. The stationary phase was activated with an aqueous 0.1% TFA solution, the loaded extract was washed with 100 μL of 50 mM NH₄HCO₃. Proteins were then reduced by addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min). Sodium betamercaptoethanol and 1% sodium dodecylsulfate (SDS) was added to the protein extract of the archaeological sample in 20 mM Tris/HCl buffer, pH 7.4 containing 50 mM NH₄HCO₃. The homogenized protein extract was washed with 100 μL of 50% (v/v) acetonitrile solution of the lyophilized protein digests were concentrated and redissolved in 0.1% trifluoroacetic acid (TFA). The aqueous solutions of the lyophilized protein digestes were concentrated and desalted by using Ultra Turrax homogenizer. After the addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% gel by Laemmli’s method. A low molecular weight calibration kit (Pharmacia) was used for estimation of the molecular weight. To increase the quality of the separation and visibility of the spots the gel was run at 4°C. Gels were stained with Coomassie brilliant blue R-250 and destained with a solution containing 5% (v/v) acetic acid and 16% (v/v) methanol.

The spots of the overexpressed proteins (compared to the healthy archaeological samples) were excised from the gel with a razorblade, placed in Eppendorf tubes, and destained by washing three times for 10 min in 200 μL of 50% (v/v) acetonitrile solution containing 50 mM NH₄HCO₃. Proteins were then reduced by 50 μL of 20 mM dithiothreitol, 100 mM NH₄HCO₃ and acetonitrile 5% for 1 h at 55°C and alkylated in 50 μL of 20 mM iodoacetamide solution. The gel pieces were dehydrated at room temperature by a Speed Vac Concentrator (Speed Vac Plus, SC100A, Savant) and covered with 10 μL of modified trypsin (Promega, Madison, WI, sequencing grade) (0.04 mg/mL). The excised spots were crushed and peptides were extracted in an ultrasonic bath (15 min) with 15 μL aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v) for 1 h at 55°C until further processing.

The bone sample with tumorous lesion was measured in eight technical replicates.

### SDS-PAGE Gel Electrophoresis and Enzymatic Digestion

100 μL of protein extract of the archaeological sample in 20 mM Tris/HCl buffer, pH 7.4 containing 3 mM EDTA, 5 mM betamercaptoethanol and 1% sodium dodecylsulfate (SDS) was homogenized by using Ultra Turrax homogenizer. After the addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% gel by Laemmli’s method. A low molecular weight calibration kit (Pharmacia) was used for estimation of the molecular weight. To increase the quality of the separation and visibility of the spots the gel was run at 4°C. Gels were stained with Coomassie brilliant blue R-250 and destained with a solution containing 5% (v/v) acetic acid and 16% (v/v) methanol.

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The bone sample with tumorous lesion was measured in eight technical replicates.

### MALDI TOF/TOF Mass Spectrometry-based Identification of the Ancient Proteins

After extraction the peptide solutions were lyophilized and redissolved in 0.1% trifluoroacetic acid (TFA). The aqueous solutions of the lyophilized protein digestes were concentrated and desalted by using Ultra Turrax homogenizer. After the addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% gel by Laemmli’s method. A low molecular weight calibration kit (Pharmacia) was used for estimation of the molecular weight. To increase the quality of the separation and visibility of the spots the gel was run at 4°C. Gels were stained with Coomassie brilliant blue R-250 and destained with a solution containing 5% (v/v) acetic acid and 16% (v/v) methanol.

The spots of the overexpressed proteins (compared to the healthy archaeological samples) were excised from the gel with a razorblade, placed in Eppendorf tubes, and destained by washing three times for 10 min in 200 μL of 50% (v/v) acetonitrile solution containing 50 mM NH₄HCO₃. Proteins were then reduced by 50 μL of 20 mM dithiothreitol, 100 mM NH₄HCO₃ and acetonitrile 5% for 1 h at 55°C and alkylated in 50 μL of 20 mM iodoacetamide solution. The gel pieces were dehydrated at room temperature by a Speed Vac Concentrator (Speed Vac Plus, SC100A, Savant) and covered with 10 μL of modified trypsin (Promega, Madison, WI, sequencing grade) (0.04 mg/mL). The excised spots were crushed and peptides were extracted in an ultrasonic bath (15 min) with 15 μL aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v) for 1 h at 55°C until further processing.

The bone sample with tumorous lesion was measured in eight technical replicates.
mass tolerance for monoisotopic peptide masses was set to 80 ppm. Carbamidomethylation was set as global modification while methionine oxidation was set as variable modification. Additionally, LID and CID fragmentation of the matched peptides were carried out for MALDI TOF/TOF to provide further evidence for the presence of the identified proteins.

**Table:**

| Position | Molecular mass (Da) | Sequence | MS/MS | Modification |
|----------|---------------------|----------|-------|--------------|
| 81-103   | 2717.24             | DVMAGLMYPPLYDAHELWHAMK |       |              |
| 193-202  | 1194.69             | TMLQMIQN     |       |              |
| 193-202  | 1251.68             | TMLQMIQN     |       |              |
| 193-202  | 1267.72             | TMLQMIQN     | Oxidation (M) | Oxidation (M) |
| 212-227  | 1760.66             | QEFQINSQGDMVDAIN |       |              |
| 233-243  | 1234.78             | VDAINECYDGY  |       |              |
| 293-302  | 1235.60             | YGKSLFHDIR   |       |              |
| 296-310  | 1791.73             | SFLHDIRNASGHYK |       | Oxidation (M) |
| 311-324  | 1716.95             | KALLAICAGDAEDY |       |              |
| 313-324  | 1475.87             | LLAICAGDAEDY |       |              |

**Figure 4:** Representative mass spectra and the list of the identified tryptic peptides of two identified tumor biomarkers. A) Annexin A10, B) Vimentin. Some keratin contamination has been detected in the sample, the tryptic peptides of keratin were used as internal calibration standards and the peaks are marked with asterisk.

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Statistical Analysis

To demonstrate the predictive value of the identified biomarkers the mass spectrometric results were statistically evaluated by ClinProTools 2.2 (Bruker Daltonics, Bremen, Germany) clustering software. Multiple spectra of the analyzed bone samples from different sample cohorts, such as osteosarcoma, non-pathological and pathological (tuberculotic) control samples were distinguished together. Recalibration, spectral alignment, peak normalization, peak detection and peak area calculation of spectra were carried out automatically by ClinProTools. A logistic regression model was performed to identify the significant predictive peaks on the basis of the normalized peak areas. Wilcoxon signed-rank test was used for non-parametric statistical analysis of the different sample cohorts.

Results and Discussion

This work focused on the identification of possible protein biomarkers of osteogenic sarcoma from a 2000-year-old anthropological sample. The proteins were separated by 1D gel electrophoresis and the interested spots were enzymatically digested (Figure 3). The tryptic peptides were analyzed by MALDI TOF/TOF MS and the identification of the resulted proteins was carried out using a PMF or MS/MS search. Based on our results several known, previously published osteosarcoma or tumor related proteins and gene products were detected from the ancient pathological bone sample (Table 3).

The identified annexins (ANXs) are calcium and phospholipid binding proteins, they play a crucial role in the exocytic and endocytic transport, regulation of cell growth, proliferation and apoptosis. It is well known, that the increased level of ANX (ANXA-10) indicates tumor progression [29,30]. The B-cell lymphoma 2-related protein A1 (BCL2A1) is a member of BCL2 proteins. BCL2A1 is responsible for the separation of pro-apoptotic BCL2 proteins. BCL2A1 shows an elevated level in case of different cancer types such as leukemia and lymphoma, also connected with autoimmunity and therapy resistance of different tumors [31–34]. Calgizzarin (S100A11) belongs to the calcium binding proteins S100 family, involved in cell growth, motility and differentiation. Calgizzarin has been correlated with tumor progression and metastasis [35,36]. DLC1 is a known tumor suppressor, acting through Rho GTPase-activating protein (RhoGAP), which is involved in the proliferation and migration of tumor cells, induces apoptosis in vitro [37–39]. Heat-shock protein’s (HSP’s) expression increases in case of thermal, physiological or other stress factors allowing the cells to survive lethal conditions. HSP’s play key role in the apoptotic and cell death process (inhibition of caspase activation). Elevated level of HSP beta-6 was found in clinical samples of patients who suffered from osteosarcoma [40,41]. DJ-1 protein is a mitogen-dependent oncogene involved in ras-related signal transduction pathway. Overexpression of DJ-1 indicates tumorous mutation [42,43]. The RhoGAP family proteins play an important role in regulating cell migration, cell morphology and cytoskeletal organization. Down regulation of RhoGAP proteins decrease the tumor suppressive effect [44]. Transferrin is a member of iron-binding blood plasma glycoproteins, that is responsible for the regulation of the free iron content in the blood. Elevated level of transferrin is correlated with tumorous diseases such as osteosarcoma [40,45]. The expression of a cytoskeletal intermediate filament protein vimentin (VIM) was also shown to increase in case of osteosarcoma [40,45–47]. VIM is considered to be a tumor biomarker, as it is promoting the metastatic spread of the tumor cells. In this study, some keratins were identified as well. The origin and the importance of these proteins are not well known, probably the identified keratins are from recent or contemporary contaminations. However, the up-regulation of cytokeratins has been published in U2OS osteosarcoma specific cell line [43].

Figure 5. ClinProTools-based Wilcoxon non-parametric statistical test of different sample cohorts. Cluster analysis from sample sets of the osteosarcoma (green), healthy control sample (red) and tuberculotic control sample (blue) groups using the peptide peaks with m/z 1180 and 1385. The x-and y-axes correspond to the relative intensities of the peptide peaks.
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Representative mass spectra and the list of the identified tryptic peptides of annexin A10 and vimentin are shown on Figure 4. The results show characteristic peaks of the identified biomarkers, however some keratin peaks are present on the spectra. This pollution is common from ancient bone tissue, but various types of keratins are upregulated in different tumors as well.

Based on our statistical analysis the different sample cohorts such as osteosarcoma, healthy control and tuberculous control could be distinguished, therefore the spectral profile of the samples with osteosarcoma is significantly different as the other two group’s profiles (Figure 5). The predictive peaks are shown in Table S1, where the statistical parameters are given for the most predictive molecular masses.

Conclusions

The aim of our study was to find potential tumor biomarkers for ancient osteosarcoma using healthy bone samples as a control. Certain proteins, which are characteristic for tumors disease can be extracted from the diseased cells, were presumably transported earlier by the blood and absorbed to the bone hydroxyapatite [48]. Using an appropriate extraction method, developed by our research group, followed by SDS PAGE, tryptic digestion and MALDI TOF/TOF, we were able to identify several proteins that were tightly connected to tumors mutations. The overexpression of annexin A10 protein, BCL-2-like protein, calzgirin, HSP beta-6, RhoGAP-activating protein 7, transferrin, and vimentin among others referred to healthy samples may indicate the presence of tumor in bones. In this study, we demonstrated that the peptide profile of the samples with osteosarcoma is statistically unique and it could be distinguished from other sample cohorts.

On the basis of our results the proteomic analyses could indicate the presence of osteosarcoma in bone tissues. Our findings showed that the well known, osteosarcoma-related clinical protein biomarkers are detectable in the investigated 2000-year-old tumorous skeletal remain. In the future, additional comparative proteomic investigations are needed for further early stage biomarker discovery of ancient primary bone cancer.

Supporting Information

Table S1 The statistical parameters of the mass spectrometric results.

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Author Contributions

Conceived and designed the experiments: LM GM GAT. Performed the experiments: AB ZP EJ CF. Analyzed the data: GM JS EJ. Contributed reagents/materials/analysis tools: LM GM. Wrote the paper: LM GAT.

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