Molecular diagnosis of paleopathological human remains by using mass spectrometry

Ph.D. Thesis

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Introduction

Osteosarcoma is the most common primary bone tumor characterized by the production of osteoid matrix from malignant cells derived from progenitor cells in the osteoblast lineage. It typically occurs in the long bones of the near metaphyseal growth plates of children and young adolescents. These mesenchymal tumor related mutations escape from the cells leading to excessive proliferation without differentiation from normal osteoblastic cells during the cell cycle. This highly aggressive neoplasm frequently metastasizes in the lungs.

Osteosarcoma has a poor prognosis because metastases are usually developed prior to clinical diagnosis. Despite intensive research for new therapies, the survival rate for patients with advanced disease still remains low.

Certain proteins, which are characteristic for tumorous disease can be extracted from the diseased cells, were presumably transported earlier by the blood and absorbed to the bone hydroxyapatite. 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. 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.

Tuberculosis is a chronic infectious disease, caused by bacteria of the *Mycobacterium tuberculosis* complex (MTC), including *Mycobacterium tuberculosis* and *Mycobacterium bovis* being the most common cause of illness in humans. Tuberculosis, one of the oldest recorded human afflictions, is still one of the most deadly and common major infectious disease today, despite the worldwide use of a live attenuated vaccine and several antibiotics.

Mycobacterium tuberculosis has the property to disseminate in the body via the blood and lymphatic systems and replicate in bones, leaving characteristic lesions in it. The most characteristic parts of the skeleton involved are the spine, hip and knee joints, the hands and the feet, but any bone can involved.

The paleoproteomic analysis can be an independent, robust, high-throughput technique for confirmation of the presents of human pathogen *Mycobacterium* species.

Aims of the study

- The extraction of ancient proteins derived from osteosarcoma and tuberculoid bone samples developing an optimized workflow.
- The separation of the extracted proteins based on their mass applying SDS gel electrophoresis.
- The mass spectrometric (MALDI TOF/TOF MS) analysis of the tryptic digested differently expressed bands (excised from the gel) compared to the control (healthy) samples.
- The identification of malignant bone tumor related molecular biomarkers and mycobacterial proteins in archaeological human skeletal remains utilizing Mascot and ProteinScape server database search engines.
- The statistical analysis of osteosarcoma, pathological (tuberculotic) and non-pathological control sample cohorts.

Materials and Methods

Archaeological bone samples

Osteosarcoma archaeological bone sample

The fragmented skeleton of a 25–35-year-old female with osteogenic sarcoma has been excavated at the Late Roman archaeological site of Szombathely (Savaria) - Szent Marton street 53, Hungary (municipal location code 6583/1) grave 186. The analyzed bone sample was collected from the cortical region of the right humerus.

The anthropological (determination of sex and age, macroscopic and morphological examinations) and paleopathological investigations (X-ray radiography) were carried out based on Knussmann (Knussmann, 1988) and Jozsa (Jozsa, 2006). A humerus from a non-cancerous skeletal remain from this cemetery (grave 199, adult female) was used for the investigations as a control sample. For further statistical validation we used our previous proteomic results of some non-pathological and *M. tuberculosis* infected bone samples.

Tuberculoid archaeological bone samples

Various *Mycobacterium tuberculosis* infected and morphologically “healthy” human skeletal remains have been analyzed from different archaeological periods. The measured paleopathological bone samples are presented in Table 1.

No.	Location	Grave	Age	Sex	Anatomy	Date
1	Bélmegyer-Csömöki domb	65	30-40	Female	vertebra	700-800 AD
2	Csongrád-Ellés	183	40-45	Male	vertebra	1000-1200 AD
3	Bácsalmás-Homokbánya	39	40-50	Male	vertebra	1500-1600 AD

Table 1: Anthropological data of the measured *M. tuberculosis* infected archaeological skeletal remains.

The paleopathological remains were investigated for DNA of *Mycobacterium tuberculosis* complex (except Csongrád-Ellés) and the test was positive in all cases. Furthermore, the presence of mycolic acids as lipid biomarkers for mycobacterial infection was described previously from these archaeological bone samples by our research group as well. The presence of mycolic acids cannot be detected in the control samples.

Extraction of ancient proteins

The bone fragments were washed with phosphate buffer saline (PBS) and distilled water to remove contaminants. Bone powder was ground by hand using pestle and 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 µl of 6 M guanidine-HCl in 0.1 M Tris (pH=7.5) at room temperature. 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 protein extract was washed with 100 µl of 2% acetonitrile in 0.1% TFA three times. The proteins were eluted by 50 µl of 50% acetonitrile in 0.1% TFA solution.

The solution was lyophilized to powder and stored at -86°C until further processing.

SDS-PAGE gel electrophoresis and enzymatic digestion

The lyophilized protein extract of the archaeological sample was dissolved in 100 µL of 20 mM Tris/HCl buffer, pH 7.4 containing 3 mM EDTA, 5 mM betamercaptoethanol and 1% sodium dodecylsulphate (SDS). After the addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min).

Sodium dodecylsulphate-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 bands the gel was run at 4ºC. 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 bands of the overexpressed proteins (compared to the healthy archaeological samples) were excised from the gel with a razorblade, were cut into three pieces and 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 NH_4HCO_3. The disulphide bonds of cysteines were then reduced by 50 µL of 20 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 and acetonitrile 5% for 1 h at 55°C. The alkylation of the cysteine groups was carried out 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) in Tris buffer (2.5 mM, pH 8.5) and left at 37°C overnight.

The digestion were stopped with 15 µL aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v). The digested samples were incubated for 60 minutes at room temperature. In the last step the peptide solutions were lyophilized.

MALDI TOF/TOF MS-based identification of the ancient proteins

After lyophilization the samples were redissolved in 0.1% trifluoroacetic acid (TFA). The aqueous solutions of the lyophilized protein digests were concentrated and desalted by using C18 ZipTip SPE pipette tips (Millipore Kft, Budapest, Hungary). Then the purified peptides were eluted directly onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by using of 3 μL of a saturated matrix solution, prepared by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/ 0.1% TFA (1/2, v/v). The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in reflectron mode for peptide mass fingerprinting (PMF) or in LIFT mode for LID (laser induced decay) and CID (collision induced decay) fragmentation. The accelerating voltage was set to 20.00 kV. The instrument uses a 337 nm nitrogen laser (model MNL-205MC, Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). Peptide masses were acquired in the range of m/z 700 to m/z 5000. Each spectrum was produced by accumulating data from at least 500 consecutive laser shots. The FlexControl 2.4 software (Bruker Daltonics, Bremen, Germany) was used to control the instrument and FlexAnalysis 2.4 software (Bruker Daltonics, Bremen, Germany) was used for spectra evaluation. Singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBInr databases (last accessed: 11/19/2012) by utilizing the MASCOT database search engine (version 2.2) (www.matrixscience.com, Matrix Science Ltd., London, UK), Bruker BioTools 3.0 software (Bruker Daltonics, Bremen, Germany) and Bruker ProteinScape server 2.1 (Bruker Daltonics, Bremen, Germany). Maximum one missed tryptic cleavage was considered, and the 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 MS to provide further evidence for the presence of the identified proteins.

Statistical analysis

Statistical analysis was carried out only 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

Osteogenic Sarcoma

This work partly 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 bands were enzymatically digested. 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 were detected from the ancient pathological bone sample (Table 1).

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 ANXs (ANXA10) indicates tumor progression. BCL2 protein family member B-cell lymphoma 2-related protein A1 (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. 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. Deleted in liver cancer 1 (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. 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. DJ1 protein produced by a mitogen-dependent oncogene involved in Ras-related signal transduction pathway. Overexpression of DJ1 indicates tumorous mutation.

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. Transferrin is a member of iron-binding blood plasma glycoproteins, which 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. The expression of a cytoskeletal intermediate filament protein vimentin (VIM) was also shown to increase in case of osteosarcoma. 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.

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|62087532	arginine/serine-rich splicing factor 6 variant	31,8	6	27,6
ARI5B_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
CAN7_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 nucleotidylexotransferase	58,4	9	22,2
MP2K6_HUMAN	Dual specificity mitogen-activated protein kinase kinase 6	37,5	7	23,4
DTNA_HUMAN	Dystrobrevin alpha	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
Q8N175_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
CAA82315	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|39653323	PHD finger protein 20-like 1 isoform 1	47,7	7	15,8
gi|39653321	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
RHG07_HUMAN	Rho GTPase-activating protein 7	122,7	10	10,9
gi|41152086	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6	42,6	9	29,3
gi|38382764	SET-binding protein isoform b	26,4	7	25,6
gi|46250431	Transcription factor NRF	77,7	11	20,4
gi|62088924	Transducin-like enhancer of split 3 splice variant 1 variant	22,9	6	37,1
gi|37747855	Transferrin	77,0	10	21,9
gi|4507659	translocated promoter region (to activated MET oncogene)	265,4	24	12,0
gi|4827050	ubiquitin specific protease 14	56,0	8	20,6
gi|71774197	ubiquitin specific protease 47	147,1	10	13,4
gi|34532272	unnamed protein product (homolog of CCDC144A protein)	56,3	12	21,9
gi|21754902	unnamed protein product (homolog of Zinc finger protein 781)	17,8	7	48,7
gi|57471648	vimentin	26,8	8	38,2
WBP4_HUMAN	WW domain-binding protein 4	42,5	7	15,7
ZN224_HUMAN	Zinc finger protein 224	82,2	10	19,4
gi|74355161	Zinc finger protein 624	85,6	11	22,5

Table 1: The identified up-regulated proteins from 2000-year-old osteogenic sarcoma. (Mascot score > 50)

Based on our statistical analysis the different sample cohorts such as osteosarcoma, healthy control and tuberculotic control could be distinguished, using two peptide peaks (m/z 1180, 1385) derived from the investigated osteogenic bone sample. The spectral profile of the samples with osteosarcoma is significantly different as the other two group's profiles (Figure 1).

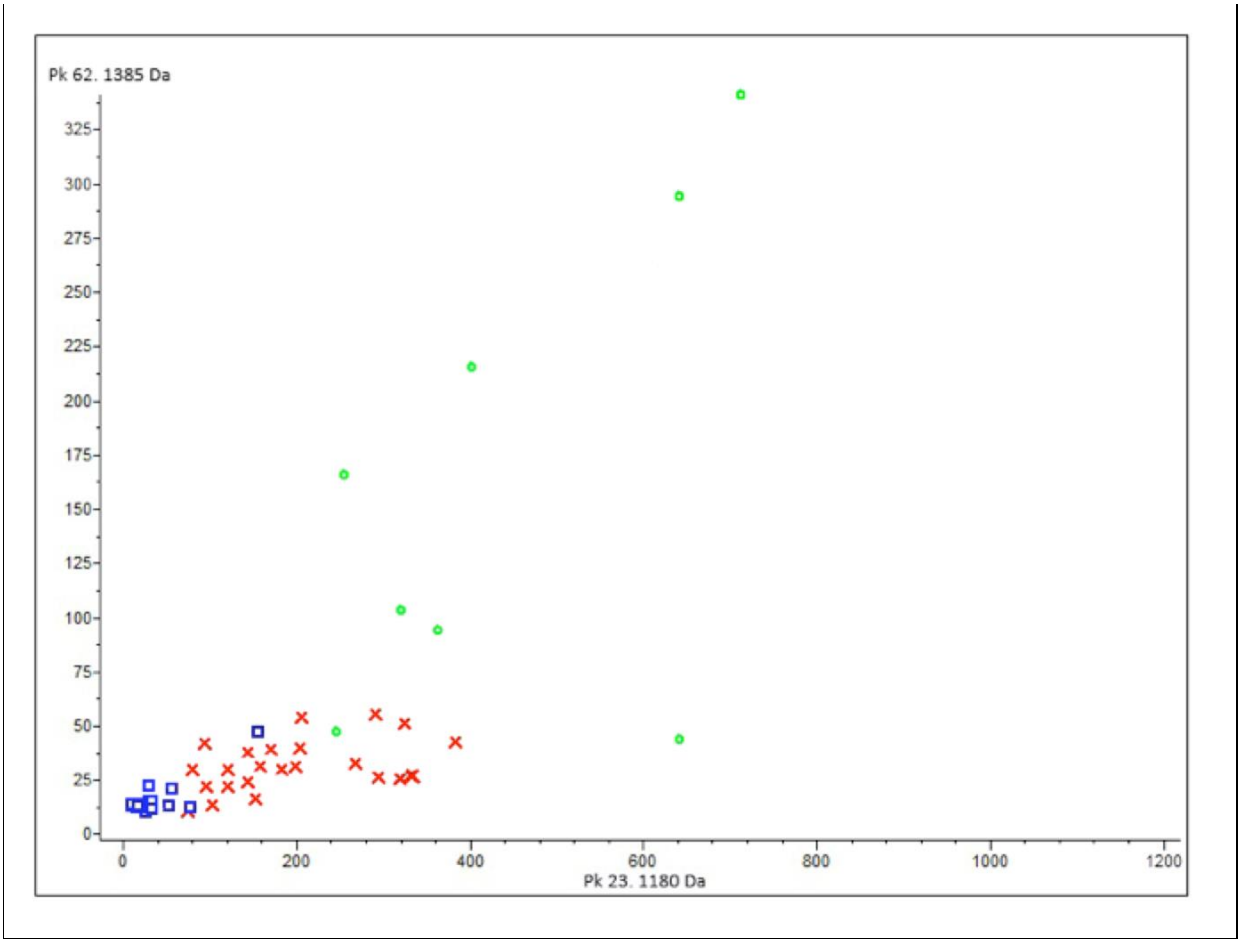

Figure 1: ClinProTools-based Wilcoxon non-parametric statistical test of different sample cohorts. Cluster analysis from sample sets of the osteosarcoma (green), healthy control sample (red) and tuberculotic control sample (blue) groups using the peptide peaks with m/z 1180 and 1385. The x-and y-axes correspond to the relative intensities of the peptide peaks.

Tuberculosis

Further target of our interest was the identification of ancient mycobacterial proteins. The protein bands were excised and digested with trypsin, and the resulting peptides were analyzed by MALDI TOF/TOF MS for protein determination. The identifications of *Mycobacterium* specific proteins using peptide mass fingerprint (PMF) analysis and direct sequencing of the tryptic peptides were successful in all investigated tuberculosis infected archaeological bone samples.

The proteomic results from the oldest investigated bone sample (Bélmegyer-Csömöki domb) have been showed in Table 2.

Protein ID	Accession no.	Theoretical MW (Da)	Mascot score	Peptides matched	Sequence coverage (%)
Adenylate kinase *(M. tuberculosis*)	gi|15607873	20113	263	14	65
Hypothetical protein (*M. tuberculosis*)	gi|254232569	50448	71	8	15
LysR family transcriptional regulator *(M. tuberculosis*)	gi|15607518	33616	68	8	29
Putative helicase *(M. tuberculosis*)	gi|260187090	111734	113	19	25
Translation initiation factor IF-2 (*M. tuberculosis*)	gi|215431780	54012	95	10	32

Table 2: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Bélmegyer-Csömöki domb grave 65.

In this sample numerous mycobacterial proteins were identified such as adenylate kinase, LysR family transcriptional regulator protein, putative helicase, translation initiation factor IF-2 protein and a hypothetical protein.

The *Mycobacterium tuberculosis* related proteomic results of the other two archaeological sites (Csongrád-Ellés and Bácsalmás-Homokbánya) have been summarized in Table 3 and 4.

In these cases several microbial enzymes (catalase-peroxidase-peroxinitritase-T katG, dehydrogenase/reductase, fumarate reductase flavoprotein subunit, glycosyl transferase, oxidoreductase, peptide sythetase nrp) as well as mycobacterial regulatory and hypothetical proteins were identified.

Protein ID	**Accession no.**	**Theoretical MW (Da)**	**Mascot score**	**Peptides matched**	**Sequence coverage (%)**
Catalase-peroxidase-peroxinitritase-T katG (*M. tuberculosis*)	gi|219557862	51220	62	8	24
Fumarate reductase flavoprotein subunit (*M. tuberculosis*)	gi|15608690	63723	163	18	34
Hypothetical protein (*M. tuberculosis*)	gi|15843166	37267	85	10	28
Oxidoreductase (*M. tuberculosis*)	gi|15610689	36808	86	10	28
Peptide synthetase nrp (*M. tuberculosis*)	gi|254233496	269241	122	23	11

Table 3: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Csongrád-Ellés grave 183.

Protein ID	Accession no.	Theoretical MW (Da)	Mascot score	Peptides matched	Sequence coverage (%)
Dehydrogenase/reductase (*M. tuberculosis*)	gi|218751933	32749	101	10	40
Fumarate reductase flavoprotein subunit (*M. tuberculosis*)	gi|15608690	63723	113	15	30
Glycosyl transferase (*M. tuberculosis*)	gi|218754716	20715	91	9	47
Hypothetical protein (*M. tuberculosis*)	gi|215428650	36324	93	12	31
Regulatory protein (*M. sp. JLS*)	gi|126436787	28284	119	8	43

Table 4: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Bácsalmás-Homokbánya grave 39.

Tuberculoid bone samples represent different eras and different regions of Hungary and that serves as an explanation for the difference between the protein profiles.

In the case of the morphologically “healthy” control samples various types of human keratins, collagens were detected, but *M. tuberculosis* proteins cannot be identified.

The suggested proteomic approach by using tandem mass spectrometric sequencing of the peptides can be feasible for species identification. All identified bacterial proteins are from human pathogen Mycobacterium species and consequently, the contamination with other e.g. soil living mycobacteria can be easily controlled and eliminated by using their different amino acid sequences. Namely, the taxa specific distinguishing of the referred and partly sequenced mycobacterial proteins can be easily carried out by using their exact amino acid sequences and the homology search of the proteins and peptides (e.g. NCBI BLAST software).

Summary of new findings

- Using an appropriate extraction method, developed by our research group, we were able to extract various, disease-related (tumor, mycobacterial infection) protein biomarkers
- The proteins, those are tightly connected to the different diseases were separated by SDS PAGE based on their mass.
- The bands of the overexpressed proteins (compared to the healthy archaeological samples) excised from the gel were digested, the tryptic peptides were analysed according to the MALDI TOF/TOF MS PMF and MS/MS mass spectra.
- Several tumor biomarker proteins (ANXA10, BCL2, DJ1, DLC1, HSP beta-6, RhoGTPase-activating protein 7, S100A11, transferrin, VIM) were successfully identified from the investigated 2000-year-old osteogenic skeletal remain based on their MS and MS/MS mass spectra utilizing Mascot database search engine.
- Osteosarcoma and tuberculosis-related proteins were identified from archaeological bone samples using MALDI TOF/TOF MS for the first time.
- *Mycobacterium tuberculosis* proteins have been described from archaeological human skeletal remains applying MALDI TOF/TOF MS and database search also for the first time.
- We demonstrated that the peptide profile of the samples with osteosarcoma is statistically unique and it could be distinguished from other sample cohorts such as non-pathological (healthy) and pathological (tuberculotic).

List of Publications

This work is based on the following articles:

1. Bona A, Papai Z, Maasz G, Toth GA, Jambor E, Schmidt J, et al. Mass spectrometric identification of ancient proteins as potential molecular biomarkers for a 2000-year-old osteogenic sarcoma. PLoS One. 2014;9(1):e87215.
Impact factor: 3.534
Citation: 1

2. Boros-Major A, **Bona A**, Lovasz G, Molnar E, Marcsik A, et al. New perspectives in biomolecular paleopathology of ancient tuberculosis: a proteomic approach. J. Archaeol. Sci. 2011;38:197–201.
Impact factor: 1.914
Citation: 8

Further publications:

Budán Ferenc, Szabó István, Jámbor Éva, **Bóna Ágnes**, Váczy Alexandra, Maász Gábor, Ohmacht Róbert, Kiss István, Márk László, Tényi Tamás: A skizofrénia biomarkereinek kimutatása és azonosítása tömegspektrometriával új lehetőséget nyithat a megelőzésben. Magyar Epidemiológia 7: p. 69. (2010)

Jarai T., Maasz G., Burian A., **Bona A.**, Jambor E., Gerlinger I., Mark L.: Mass spectrometry-based salivary proteomics for the discovery of head and neck squamous cell carcinoma, Pathol. Oncol. Res. 18(3): 623-628 (2012)
Impact factor: 1.555
Citation: 18

Szabo Gy. T.; Tihanyi R.; Csulak F.; Jambor E.; **Bona A.**; Szabo Gy.; Mark L.: Comparative salivary proteomics of cleft palate patients, The Cleft Palate-Craniofacial Journal 49(5): 519-523 (2012)

Impact factor: 1.238

Citation: 4

Szanto I., Mark L., **Bona A.**, Maasz G., Sandor B., Gelencser G., Turi Zs., Gallyas F.: High-throughput screening of saliva for early detection of oral cancer.: A pilot study, Technology in Cancer Research and Treatment 11(2): 181-188. (2012)

Impact factor: 1.943

Citation: 9

Gubicskóné Kisbenedek Andrea; Kovács Bernadett; Polyák Éva; Szekeresné Szabó Szilvia; Breitenbach Zita; Szabó Zoltán; Márk László; **Bóna Ágnes**; Figler Mária: A bogyós gyümölcsökből készült készítmények Rezveratrol-, rutin- és kvercetintartalmának meghatározása Új Diéta. - ISSN 1587-169X. - 2012. 19. évf. 5-6. sz., p. 34-35.

Jambor E.; Bona A., Schmidt J., Mark L., Ohmacht R.: Preparation and investigation of LC packing made by microwave-assisted solid-phase synthesis. Journal of Separation Science 36 (5) 827–831 (2013)

Impact factor: 2.594

Citation: 0

Kisbenedek G. A., Szabo Sz., Polyak E., Breitenbach Z., **Bona A.**, Mark L., Figler M.: Analysis of trans-resveratrol in oilseeds by high performance liquid chromatography, Acta Alimentaria (2013)

Impact factor: 0.427

Citation: 0

Total impact factor: 13.205

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