Data independent acquisition-mass spectrometry (DIA-MS)-based comprehensive profiling of bone metastatic cancers revealed molecular fingerprints to assist clinical classifications for bone metastasis of unknown primary (BMUP)

Xin Ku, Chunlin Cai, Yan Xu, Su Chen, Zhenhua Zhou, Jianru Xiao, Wei Yan

1Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China; 2Department of Orthopedic Oncology, Shanghai Changzheng Hospital, The Second Military Medical University, Shanghai 200003, China

Contributions: (I) Conception and design: W Yan, J Xiao; (II) Administrative support: W Yan, J Xiao; (III) Provision of study materials or patients: W Yan, J Xiao; (IV) Collection and assembly of data: S Chen, Z Zhou; (V) Data analysis and interpretation: X Ku, Y Xu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Dr. Xin Ku. Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China. Email: xinku@sjtu.edu.cn; Prof. Wei Yan. Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China. Email: weiyan_99@uwalumni.com, weiyan@sjtu.edu.cn; Prof. Jianru Xiao. Department of Orthopedic Oncology, Shanghai Changzheng Hospital, The Second Military Medical University, Shanghai 200003, China. Email: jianru Xiao83@163.com.

Background: Bone metastasis is the third most common metastatic cancers worldwide. It is a group of highly heterogeneous diseases with various potential cancer primaries. Among them, one third was diagnosed as bone metastasis of unknown primary (BMUP) due to lack of indication for the primary tumor even after comprehensive examinations. Thus, the prognosis of BMUP is often very poor since the treatment was largely empirical and untargeted. To assist identification of the primary tumor, a series of molecular markers including traditional tissue-specific histochemistry as well as gene and mRNA markers were developed with moderate to good sensitivity and specificity.

Methods: In this paper, we carried out a comprehensive expression profiling for fresh-frozen tissue samples of bone metastasis from lung, prostate and liver cancers using high resolution, data-independent-acquisition mass spectrometry (DIA-MS). The proteome variation was analyzed and protein classifiers were prioritized.

Results: Over 6,000 proteins were quantified from 18 samples, which, to the best of our knowledge, was never achieved before. Further statistical analysis and bioinformatics data mining revealed 4 significant proteins (RFIP1, CK15, ESYT2, and MAL2) with excellent discriminating capabilities with AUCs higher than 0.8.

Conclusions: The comprehensive proteome map of bone metastases will complement available genomic and transcriptomic data. Newly discovered protein classifiers will expand current diagnostic arsenal for tissue of origin studies in BMUP. Furthermore, the proteome map generated in this study by DIA-MS allows future data re-mining as our knowledge advances to assist investigation of bone metastasis and progression of tumors as well as the development of diagnostic tools and prognosis management for BMUPs.

Keywords: Bone metastasis of unknown primary (BMUP); proteomic profiling; data independent acquisition; biomarker; fresh-frozen tissue

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Introduction
Cancer of unknown primary (CUP) is a heterogeneous metastatic carcinoma in the absence of an identifiable primary neoplasm. It is estimated that about 15% of all cancer patients were diagnosed for the first time due to the clinical metastatic syndromes. The primary tumor for the majority of these patients could be determined after standardized diagnostic work-ups including a thorough clinical history, physical examination, biopsies, and imaging studies (CT, MRI, PET) (1,2). However, still one third of all metastatic cancers with tumor origins remain elusive, which account for 3–10% of total cancer diagnoses worldwide (3-7). The peak incidence was at around 70 years of age and was slightly higher in males than in females (8-10). Although incidence has been declining since 1990s attributed to the diagnostic advances, CUP still remains the fifth leading cause of cancer-related death worldwide (3,11,12). Due to lack of indication of tumor origins, the treatment was largely empirical by utilizing taxane or platinum-based agents, which is usually associated with bad responses and resulted in poor prognosis (13-15). For this group, the median overall survival is only 6–9 months and less than 50% of 1-year overall survival (7,16).

Bone is the third most frequent site where metastases from primary tumor could develop after lung and liver (17-19). Over the past decades, average survival of patients with bone metastasis from lung cancer was dishearteningly 4–8 months (20,21). Treatment decisions are currently based on histology classifications of cancer primary. However, among patients with bone metastasis, 20–30% still have no indication of the primary tumor after comprehensive examinations (22,23). The etiology of this disease remains unknown. For these patients in routine clinical practice, the main strategy for identifying the primary tumor was tissue-of-origin (TOO) studies using histochemistry and immunohistochemistry (IHC), which were considered as standard tests to suggest the origin of the lesion. Tissue-specific protein markers such as cytokeratins (CKs) including CK7 (KRT7) and CK20 (KRT20), homeobox protein Nkx-2.1 (TTF1), mammaglobin-A, and homeobox protein CDX-2 have been widely applied to predict primary sites from lung cancer, prostate cancer, and liver cancer. The disease tissues were from patients that were firstly diagnosed with bone metastatic cancer, aiming for deepening our insight into the molecular fingerprints of bone metastatic cancers which will be of significance for guiding TOO elucidation and prognosis management.

Methods

Patient cohort
Informed consent forms were received from all patients included in this study, and all experimental work in this study was approved by the Review Board and Ethical Committee of Shanghai Changzheng Hospital, affiliated to The Second Military Medical University.

A total number of eighteen freshly frozen tissue samples were obtained from Shanghai Changzheng Hospital Tissue BioBank, encompassing bone metastatic carcinoma tissues with primary sites from lung cancer, prostate cancer, and liver cancer. The disease tissues were from patients that were firstly diagnosed with bone metastatic cancers and underwent surgery without any pre radio- or chemotherapy. The primary tumor site was identified after comprehensive
examination including advanced imaging techniques and IHC analyses.

**Tissue homogenization and protein extraction**

After carefully reviewing all the sample information, the obtained bone metastatic tissue blocks were homogenized and lysis buffer was added into each sample. The lysis buffer contains 0.2% acid labile surfactant (ALS) in 20 mM HEPES buffer with 1× protease inhibitor (Roche, Basel, Switzerland). All the sample were homogenized in liquid nitrogen. The homogenates were then incubated for 30 minutes on ice. After that, the lysates were spun down at 20,000 g for 30 min at 4 °C. All the protein concentrations were determined by standard BCA assay.

**Protein digestion and peptide purification**

Urea (6M) was used to denature the proteins at room temperature for 1h. Then the proteins were reduced with 5 mM tris (2-carboxyethyl)phosphine (TCEP) and incubated at room temperature for 0.5 h. Iodoacetamide (IAA, 6.25 mM) was added to alkylate the protein for 0.5 h at room temperature at dark. The mixture was then diluted with 6 volumes of 50 mM ammonium bicarbonate buffer and digested using sequence modified trypsin (Promega, Madison, WI, USA) at an enzyme-to-substrate ratio of 1:100 (w/w), for 12 hours at 37 °C. Each digested protein solution was quenched with 1 uL phosphoric acid and the pH was adjusted to 2. Then the acidified mixture was loaded onto a 96-well C-18 cartridge (ThermoFisher, USA) and washed 3 times with 0.1% formic acid. After that, the desalted peptides were eluted with 50% ACN and dried under vacuum.

**Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis**

The dried peptides were dissolved in 0.1% formic acid (0.5 µg/µL). iRT kit (Biognosys, Switzerland) was added according to manufacturer’s instruction prior to LC-MS/MS analysis. LC-ESI-MS/MS was performed by coupling a nanoLC (Dionex Ultimate 3000, ThermoFisher Scientific) to an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific). For each analysis, 2 µL of dissolved peptides was delivered to an analytical column (Dikma, inspire C18, 3 µm, Canada, 150 mm × 75 µm, self-packed) and separated using a 62 min gradient from 7% to 35% of solvent B (0.1%
formic acid in acetonitrile) at 300 nL/minute flow rate.

For spectral library generation, an Orbitrap Fusion mass spectrometer was operated in data dependent acquisition (DDA) mode, automatically switching between MS and MS/MS. Full scan MS spectra (350–1,550 m/z) were acquired in the Orbitrap at 60,000 resolution (at m/z 400) after accumulation precursor ions to a target value of 200,000 for a maximum time of 100 ms. Internal lock mass calibration was enabled using the ion signal (Si(CH₃)₂O)₆H⁺ at m/z 445.120025 present in ambient laboratory air. Tandem mass spectra were recorded for maximum 3 seconds by higher energy collision induced dissociation (HCD, target value of 10,000, max 35 ms accumulation time) at a normalized collision energy of 30% in the orbitrap. To maximize the number of precursors targeted for analysis, dynamic exclusion was enabled with one repeat count in 60 s exclusion time.

DIA analysis was performed with the same mass spectrometer and LC system as DDA. The same analytical column, same gradient, same flow rate and buffers were used. The mass spectrometer was operated in t-MS2 mode. The duty cycle started from a high-resolution MS1 scan event acquired at a resolution of 60,000 and followed by 30 DIA scans with 21 m/z isolation windows (1 m/z overlapped between each window) at 30,000 resolution covering the precursor mass range from m/z 400 to 1,000 over the entire chromatographic elution profile. The rest of the acquisition parameters remained identical to the DDA analyses.

**Spectral library construction and data analysis**

The spectral library was built up by combination of 10 DDA files (acquired from different tissue subtypes), the peaks lists from these files were picked from raw MS data files and were then searched against Uniprot Human Protein Database (2016.09.16) using Proteome Discoverer version 1.4 (Thermo Fisher Scientific). The search was performed considering carbamidomethylation of cysteine residues as fixed modification and methionine oxidation as variable modifications. Trypsin was specified as the proteolytic enzyme and up to two missed cleavages were allowed. The mass tolerances were set to 10 ppm for the precursor ions and 0.02 Da for the fragments. All peptides were filtered at high confidence level. The protein false discovery rate, which was determined by a target-decoy search strategy, was set to 1%. The spectral library was generated from DDA data using Spectronaut (Biognosys, Switzerland) with a FDR cutoff set to 0.01. The assay fragments were
restricted from 3 to 6, and fragments were selected from m/z 300–1800. All DIA raw data files were imported in Spectronaut after being converted in HTRMS Converter (Biognosys, Switzerland). Peptide fragment ion peak areas were extracted by searching against above-mentioned library. Retention time prediction type was set to dynamic iRT and correction factor of 1. Cross run normalization was enabled for systematic variance correction of the LC-MS performance and local normalization strategy was used based on the assumption that although a small fraction of peptides will be up or down regulated, the total quantity of all the peptides within the sample will remain equal across runs and along retention time due to the equal amount of protein sampling guided by BCA assay. All results were filtered by a Q value cutoff of 0.01 (corresponds to a FDR of 1%). Peptide intensity was calculated by summing the peak areas of their respective fragment ions for MS2 and protein intensity was calculated by summing the intensities of their respective peptides. Significance analysis of protein abundance changes was calculated using two-side t-test. Further data interpretation and functional annotation were performed using DAVID, v6.8, Ingenuity Pathway Analysis (IPA), R and online tool powered by R language (http://www.omicsolution.org/wu-kong-beta-linux/main/).

**Results**

**Overview of patient proteomic profiles**

As described in Figure 1, we collected three most common bone metastatic cancer tissues from each patient in the population (n=18), with primary tumors located in lung (n=7), prostate (n=3), and liver (n=8).

Our proteomic workflow followed the general sample preparation procedure, via tissue homogenization in liquid nitrogen, protein alkylation and digestion. Each fresh-frozen specimen was homogenized and lysed using a simplified and highly efficient “precipitation-free” protein extraction protocol as described before. Detergent-free, ALS were utilized in the lysis buffer, since ALS can degrade easily under acidic conditions which is perfect to be coupled to proteomics pipelines. Resulted peptides were purified via 96-well C18 plates and delivered to a nanoLC coupled with high resolution mass spectrometer (Orbitrap Fusion). Three samples from the cohort (from each primary location category) were used for data independent acquisition (DDA) to establish a comprehensive spectral library. All samples were subjected to LC-MS/MS individually for generation of a quantitative “proteome map” for each single patient via data independent acquisition mass spectrometry (DIA-MS). The raw data were processed and quantified proteins were further analyzed with bioinformatics tools.

The samples were divided into 3 subgroups according to their diagnostic tumor primary: 7 with primary lung cancer (BM_Lung), 3 with primary prostate cancer (BM_Prostate) and 8 with primary prostate cancer (BM_Liver). After protein quantification and data normalization (see methods), more than 6000 proteins were quantified (Figure 2A) from 18 DIA-MS runs at q-value less than 0.01, each in a 62-minute elution gradient. The majority of identified proteins were shared among different cancer tissues. With 527 proteins exclusively identified in patients with bone metastases from liver, this group showed the highest number of proteins identified in their proteomic profiles. There are 741 proteins that were found in patients with bone metastases from liver and lung, but not prostate; 675 proteins were shared by patients with bone metastases from liver and prostate, but not lung. Principle component analysis (PCA) on all identified proteins from all samples reveals no obvious batch effects between samples (Figure 2B). Gene ontology (GO) analysis on shared proteins suggested that these proteins mainly located in cytoplasm and extracellular exosomes, and participated in molecular functions such as cell adhesion and oxidation-reduction processes, many of which are vital activities taken place in tumorigenesis and metastasis (Figure 2C).

**Tissue-specific proteome profiles**

Current cancer treatment strategies were mainly based on histological classifications of cancer tissues. Therefore, treatment options for CUP patients are very much dependent on TOOs studies. Some reported molecular classifiers from gene/mRNA expression studies are able to predict primary of cancer tissues, but to the best of our knowledge, there are no systematic investigation on proteomic signatures of bone metastatic cancers. To uncover potential tissue-specific proteome profiles, we further applied statistical analysis (student’s t-test, double sided, P<0.01) on their expression profiles of all proteins, shortlisted protein candidates for each origin category (BM_Lung/Prostate/Liver) and annotated their roles in terms of cellular location, biological processes, and molecular functions (Figure 3). Most quantified proteins were from cytosol or cytoplasm, for BM_Lung/Prostate groups, considerable proteins were also identified from nucleus/
nucleoplasm, indicating potential active transcriptional regulations in the cancer cells of these tissues. As expected, the top enriched biological process for BM_liver group is oxidation-reduction process. While for BM_liver/prostate groups, transcription regulation and cell division are the top two enriched processes. Most of the tissue-specific proteins were found to be binding proteins in terms of molecular function, to interact with metal ions (Zinc), kinases, ATPs, RNAs, chromatins and so on.

**Protein marker panel to differentiate bone metastasis from lung, prostate and liver cancers**

Systematic analyses of known primaries provide tumor-specific gene signatures for classifying cancers which were reported in many studies with high accuracy (52-54). Many of these gene classifiers have been applied successfully to assist TOO prediction and diagnosis. Based on the aforementioned tissue-specific proteome signature proteins, we further probed the possibility to generate a protein marker panel to differentiate the three metastatic cancer groups (BM_Liver/Prostate/Lung). Analysis of variance (ANOVA) and orthogonal partial least square-discriminant analysis (OPLS-DA) was applied which led to 30 proteins with significance (P<0.01, Figure 4A). PCA clearly showed that each group represented a distinct cluster from the rest groups on PC1 or PC2 dimension, indicating the obvious variations of marker protein expressions among three groups (Figure 4B). Heat map using 30 significant proteins revealed details on protein abundance among three groups (normalized abundance, Figure 4C). Unsupervised clustering also gave acceptable grouping results, showing four subgroups representing BM_Liver (purple), BM_Liver/Prostate (cyan), BM_Prostate/Lung (green), and BM_Lung (red) (Figure 4D). There is a high likelihood that interference from prostate samples gave rise to the internal split of both BM_Lung and BM_Liver groups, which resulted in 4 individual subgroups. Network analysis showed alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) family members are significant molecules in aberrant metabolic pathways across many cancers as reported (Figure 4E) (55,56).

To further narrow down the candidate list to obtain a more practical panel for clinical applications, we applied regression analysis and assess the performance of these classifiers by plotting their receiver operating characteristics.
Four protein classifiers, RFIP1, KRT15, ESYT2 and MAL2, were finalized to distinguish bone metastasis from liver and lung cancer (prostate samples were excluded due to small sample size). These markers displayed very good sensitivity and specificity to differentiate two tumor primaries, with areas under the curves (AUC) higher than 0.8 (Figure 5B). Their expression patterns across different primaries were plotted in Figure 5C. CK family members (KRT7 and KRT20) were existing tumor markers for IHC analysis in clinically practice. In this study, another CK family member KRT15 (CK15) has achieved the highest AUC of 0.93, indicating the strong capability of this molecule to distinguish tumor origins from lung and liver.

Discussion

Bone metastatic cancers showed highly heterogeneous morphology and clinical outcomes. The rarity of this tumor around the globe has resulted in poor understanding of the tumor biology and etiology. This study carried out proteomic profiling of individual bone metastatic cancer...
Figure 3 Tissue-specific proteome profiles. Statistical analysis shortlisted protein candidates for each origin category (BM_Lung/Prostate/Liver) and annotated their roles in terms of (A) cellular location, (B) biological processes, and (C) molecular functions. Results showed that most quantified proteins were from cytosol or cytoplasm, for BM_Lung/Prostate groups, considerable proteins were also identified from nucleus/nucleoplasm, indicating potential active transcriptional regulations in the cancer cells of these tissues. As expected, the top enriched biological process for BM_liver group is oxidation-reduction process. While for BM_liver/prostate groups, transcription regulation and cell division are the top two enriched processes. Most of the tissue-specific proteins were found to be binding proteins in terms of molecular function, to interact with metal ions (Zinc), kinases, ATPs, RNAs, chromatin and so on.
Figure 4 Protein classifier panel to differentiate bone metastasis from lung, prostate and liver cancers. Analysis of variance (ANOVA) and orthogonal partial least square-discriminant analysis (OPLS-DA) was applied which led to 30 proteins with significance (A). PCA clearly showed that each group represented a distinct cluster from the rest groups on PC1 or PC2 dimension, indicating the obvious variations of marker protein expressions among three groups (B). Heat map using 30 significant proteins revealed details on protein abundance among three groups (C). Unsupervised clustering also gave acceptable grouping results, showing four subgroups representing BM_Liver (purple), BM_Liver/Prostate (cyan), BM_Prostate/Lung (green), and BM_Lung (red) (D). Network analysis (E) showed alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) family members are significant molecules in aberrant metabolic pathways across many cancers as reported.

patients, using 18 fresh frozen samples across different cancer primaries, including lung, prostate and liver. To the best of our knowledge, this is the first comprehensive proteomic study with identification of over 6,000 proteins, which were associated with a vast area of cellular activities such as inflammation and cell adhesion. It is exciting that two plasma membrane proteins, ESYT2 and MAL2, were found with good differentiating performance, which could act as cell surface markers to facilitate further research on bone metastatic cancer primary studies and the many further uses that can be envisaged.

The more comprehensive picture of the proteomic profile of bone metastases will complement available genomic and transcriptomic data, in the meantime enable navigation of patient proteomes, which will deepen our understanding concerning cancer metastasis development and progression. The generated digital, re-minable “proteome map” by next generation data independent acquisition mass spectrometry for each individual sample provided fundamental information to characterize the landscape of the proteome variations for bone metastatic cancers, which will provide biological insight and hold considerable promise to accelerate the development of novel markers for precise diagnosis of bone metastasis of unknown primary (BMUP).

Further experiments concerning extending the scope of bone metastatic cancer primaries including breast cancers, gastrointestinal cancers, kidney cancers as well as thyroid cancers are currently ongoing. With more upcoming proteomics data acquired from bone metastatic patient samples in the future, we could conduct big data analysis eventually, which will deepen our insight into the molecular fingerprints of BMUPs and guide the therapeutic and prognostic management.

Conclusions

We characterized proteome landscape for individual bone
Figure 5 Four classifier panel to differentiate bone metastasis from liver and lung. Regression analysis was performed to shortlist four protein classifiers and the performance of these classifiers was evaluated by plotting their receiver operating characteristics (ROC) curves in (A). Four protein classifiers, RFIP1, KRT15, ESYT2 and MAL2, were finalized to distinguish bone metastasis from liver and lung cancer (prostate samples were excluded due to small sample size). These markers displayed very good sensitivity and specificity to differentiate two tumor primaries, with areas under the curves (AUC) more than 0.8 (B). Their expression patterns across different primaries were plotted in (C).

metastasis patient tissues. Compared to previous proteomic studies, it is the first time that proteome signatures of fresh-frozen samples with more than 6,000 newly identified proteins which covered various cellular locations and biological processes. Systematic analysis of patient proteome revealed four novel and significant protein classifiers, two of which were cell surface proteins. Furthermore, the permanent proteome map generated in this study by DIA-MS allows future re-analysis and re-mining of data in silico as our knowledge advances, which is extremely practical for precious and nonrenewable clinical specimen, to assist investigation of bone metastasis and progression of tumors as well as the development of diagnostic tools and prognosis management for BMUPs.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.03.41). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are
appropriately investigated and resolved. Informed consent forms were received from all patients included in this study, and all experimental work in this study was approved by the Review Board and Ethical Committee of Shanghai Changzheng Hospital, affiliated to The Second Military Medical University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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