Genomics Proteomics Bioinformatics xxx (2018) xxx–xxx

HOSTED BY

ELSEVIER

Genomics Proteomics Bioinformatics

www.elsevier.com/locate/gpb
www.sciencedirect.com

LETTER

A Systems Biology Approach for Studying Heterotopic Ossification: Proteomic Analysis of Clinical Serum and Tissue Samples

Erin L. Crowgey [1,a], Jennifer T. Wyffels [2,3,b], Patrick M. Osborn [4,c], Thomas T. Wood [4,d], Laura E. Edsberg [2,*,e]

[1] *Nemours Biomedical Research, Nemours Alfred I. duPont Hospital for Children, Wilmington, DE 19803, USA*
[2] *Natural and Health Sciences Research Center, Center for Wound Healing Research, Daemen College, Amherst, NY 14226, USA*
[3] *Department of Computer and Information Sciences, Center for Bioinformatics and Computational Biology, University of Delaware, Newark, DE 19711, USA*
[4] *San Antonio Military Medical Center, San Antonio, TX 78219, USA*

Received 8 February 2018; revised 28 March 2018; accepted 16 April 2018
Available online xxxx

Handled by Heng Zhu

KEYWORDS

Heterotopic ossification;
Proteomics;
Runt-related transcription factor 2;
Extracellular matrix organization;
Keratinization

Abstract **Heterotopic ossification** (HO) refers to the abnormal formation of bone in soft tissue. Although some of the underlying processes of HO have been described, there are currently no clinical tests using validated biomarkers for predicting HO formation. As such, the diagnosis is made radiographically after HO has formed. To identify potential and novel biomarkers for HO, we used isobaric tags for relative and absolute quantitation (iTRAQ) and high-throughput antibody arrays to produce a semi-quantitative **proteomics** survey of serum and tissue from subjects with (HO^+) and without (HO^-) heterotopic ossification. The resulting data were then analyzed using a systems biology approach. We found that serum samples from subjects experiencing traumatic injuries with resulting HO have a different proteomic expression profile compared to those from the matched controls. Subsequent quantitative ELISA identified five blood serum proteins that were differentially regulated between the HO^+ and HO^- groups. Compared to HO^- samples, the amount of insulin-like growth factor I (IGF1) was up-regulated in HO^+ samples, whereas a lower amount of osteopontin (OPN), myeloperoxidase (MPO), **runt-related transcription factor 2** (RUNX2),

* Corresponding author.
E-mail: ledsberg@daemen.edu (Edsberg LE).
[a] ORCID: 0000-0002-2037-0389.
[b] ORCID: 0000-0003-0680-5419.
[c] ORCID: 0000-0002-0115-2546.
[d] ORCID: 0000-0002-2744-6905.
[e] ORCID: 0000-0002-0592-3045.

Peer review under responsibility of Beijing Institute of Genomics, Chinese Academy of Sciences and Genetics Society of China.

https://doi.org/10.1016/j.gpb.2018.04.006

Please cite this article in press as: Crowgey EL et al, A Systems Biology Approach for Studying Heterotopic Ossification: Proteomic Analysis of Clinical Serum and Tissue Samples. Genomics Proteomics Bioinformatics (2018), https://doi.org/10.1016/j.gpb.2018.04.006

and growth differentiation factor 2 or bone morphogenetic protein 9 (BMP-9) was found in HO^+ samples (Welch two sample *t*-test; $P < 0.05$). These proteins, in combination with potential serum biomarkers previously reported, are key candidates for a serum diagnostic panel that may enable early detection of HO prior to radiographic and clinical manifestations.

Introduction

Heterotopic ossification (HO), the abnormal formation of mature lamellar bone in nonosseous (soft) tissue, is a significant problem for wounded soldiers that have survived high energy blast injuries [1,2]. A recent study on soldiers from Operation Enduring Freedom and Operation Iraqi Freedom reveals that the highest risk of HO follows amputation from a blast mechanical injury, with HO accounting for >60% combat-related extremity injuries [1,3]. Of interest, in the military population, formation of HO is associated with chronic pain, prostheses not fitting properly, joint ankylosis, functionality limitations, longer rehabilitation, and substantial morbidity [3]. Additionally, HO occurs post-trauma in elective hip arthroplasty, externally fixed distal humerus fractures (42%), spinal cord injury (SCI), and closed brain injury in civilian populations [4].

Treatment regimens for HO are limited by a lack of understanding of the cellular events that contribute to disease onset. Although non-steroidal anti-inflammatory (NSAID) drugs and radiation therapy used prophylactically can be effective as a treatment for HO, many patients need at least one surgical excision of ectopic bone [5]. Multiple diagnoses, including hemostasis and polytrauma, often present in combat casualties, make these prophylactic treatments contraindicated, and currently there are no pharmaceutical treatments yet approved by the United States Federal Drug Administration to treat HO once present [5].

Recent technological advancements in the field of mass spectrometry (MS) have enhanced the ability to perform proteomic analysis of biological samples and facilitate the identification of disease biomarkers [6]. High-throughput MS techniques, such as isobaric tags for relative and absolute quantitation (iTRAQ), enable a global analysis of the proteome differences between biological samples. This approach enables a wholistic data driven experimental design that does not require *a priori* specification of protein targets. The objective of this study was to collect and integrate serum and tissue proteomes from HO^+ and HO^- subjects, in order to identify proteins and pathways that are dysregulated in the disease state and provide insight into potential biomarkers for early disease detection and monitoring.

Results

Subject demographics and experimental workflow

Forty-four subjects were enrolled in this study. Tissue samples were collected from 42 subjects with 41 tissue samples having matched serum samples. HO^- subjects ($n = 33$) aged 22–83 years, whereas HO^+ subjects ($n = 10$) aged 22–40 years. The HO^- tissue samples were acquired mainly through total hip arthroplasty, whereas the HO^+ samples were acquired via hip revision or HO excision (**Table 1**). Serum and tissue samples were analyzed following the workflow shown in **Figure 1**.

High-throughput screening and Western blot validation

To identify potential markers for HO, high-throughput antibody microarrays were used for an initial screening of 877 cell signaling proteins by comparing the HO^- and HO^+ groups. >200 protein candidates had a 50% or greater difference in spot intensity between the pooled HO^- and pooled HO^+ serum samples. These 200 candidates were further filtered to remove proteins with high variations for duplicate measurements, flagged protein spots with irregular margins, or proteins with a global normalized score <800. As a result, 67 targets were retained for further validation and the top 18 proteins based on Z-ratios were subjected to Western blotting analysis.

Western blots validated the microarray data for phosphorylated GRB2-associated-binding protein 1 (Gab1 Y627) and apoptosis regulator BAX between the pooled HO^+ and pooled HO^- samples. However, spot intensity was weak for both Gab1 Y627 and BAX, and the BAX antibody had strong non-specific cross reactivity. Weak binding and cross-reactivity in addition to large sample volume requirements diminished the utility of Western blotting and as a result no additional serum samples were analyzed using this technique.

Table 1 Subject demographics

	HO^-			HO^+		
	M	F	Age range (mean)	M	F	Age range (mean)
Subjects						
With serum samples	18	13	22–83 (54)	9	1	22–40 (29)
With tissue samples	20	13	22–83 (52)	8	1	22–40 (28)
Injury etiology						
Total hip arthroplasty	11	8	28–83 (59)			
Open reduction and internal fixation	6	3	25–64 (45)			
Hip revision		3	45–62 (56)	1	1	36–40 (38)
HO excision				8		22–31 (26)
Others	3		22–36 (29)			

Note: HO, heterotopic ossification; M, male; F, female.

Please cite this article in press as: Crowgey EL et al, A Systems Biology Approach for Studying Heterotopic Ossification: Proteomic Analysis of Clinical Serum and Tissue Samples. Genomics Proteomics Bioinformatics (2018), https://doi.org/10.1016/j.gpb.2018.04.006