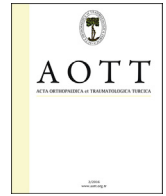




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Gene markers of fracture healing in early stage and the regulatory mechanism during the process using microarray analysis

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ABSTRACT

Background: The aim of this study was to explore crucial markers and uncover the regulatory mechanisms of fracture healing in the early stage.

Methods: Gene expression profile of GSE45156 was downloaded, in which 3 fractured samples and 3 unfractured samples were used in our present study. Based on the threshold value, differentially expressed genes (DEGs) were selected between two kinds of samples using limma package in R. Enrichment analysis of these DEGs was performed by DAVID software. Furthermore, protein–protein interaction (PPI) network was established integrating information in STRING database, and visualized by Cytoscape software.

Results: We identified a set of 960 DEGs including 509 up-regulated and 451 downregulated genes. Biological processes involving RNA splicing and cell cycle were significantly enriched for the up-regulated genes such as Snrpd2, Eftud2, Plk1 and Bub1b, whereas skeletal system development and bone development processes were predominant for down-regulated genes like Ubc. In the constructed PPI network, all the five genes were the predominant nodes, of which Snrpd2 was linked to Eftud2, while Bub1b was to interact with Plk1.

Conclusion: Five candidate genes crucial for indicating the process of fracture in early stage were identified. Eftud2, Snrpd2, Bub1b and Plk1 might function through the involvement of cell-cycle-related BP, while Ubc might influence the protein degradation during bone development. However, more experimental validations are needed to confirm these results.

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Introduction

Fracture is a serious problem worldwide and nearly 10% of all the fractures will develop into nonunion. The risk factors might be the inadequate functional stability, excessive soft tissue damage and infection.¹ Fracture healing is commonly considered as a complex process that involves the coordinated action of multiple proteins and genes to repair the structural integrity without the formation of scar.² Although standard clinical management of fracture healing exists, patients with extensive trauma would take a long duration to union, which might cause substantial morbidity and poor outcomes.³

Initial inflammatory response, generation of soft and hard callus, bone union and bone remodeling are the primary events during the repair.⁴ Numerous cell types are involved during the fracture healing including hematopoietic cells, which are essential for hematoma formation and the inflammatory response, as well as endothelial cells.^{5,6} Mesenchymal cells are another crucial cell type whose proliferation and differentiation contribute to the bone generation in the process of repair.⁷ The pluripotent mesenchymal progenitors are able to differentiate to osteoblasts or chondrocytes.⁸ In severe fractures with substantial soft tissue injury, mesenchymal progenitors can also facilitate to fracture healing.⁹ Due to the significant role of mesenchymal cells in fracture healing, multiple studies have been investigated to explore markers of this kind of cell. Among the established markers, alpha smooth muscle actin (α SMA) was identified as a marker of the mesenchymal progenitor cell facilitating to osteochondral events in fracture healing by a recent study.¹⁰ Moreover, the study labeled the

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α SMA-expressing cell populations which were involved in fracture repair, and further examined their expression profiles in the early stage of fracture healing. Though samples of 2 days and 6 days after fracture were both contained, their emphasis was obviously on 6 days, and consequently they found the increased genes relating to matrix production and remodeling as well as the decreased genes involving in Notch signaling process. However, crucial genes in early stage (2 days) after fracture as well as the potential regulatory interactions between them were scarce.

Therefore, we re-analyzed the microarray data of GSE45156 deposited by Matthews et al.¹⁰ and just utilized the samples of 2 days after fracture, to investigate the gene alterations in the initial of the fracture and the corresponding repair process. Bioinformatics methods such as differential analysis, functional enrichment analysis and protein–protein interaction (PPI) network were performed in our study, to explore the crucial gene markers and uncover the underlying regulatory mechanisms during the early stage of fracture healing.

Methods

The study did not involve any human or animals, so the ethical approval was not required.

Microarray data resource

Gene expression profile of GSE45156, which was deposited by Matthews et al.¹⁰ in the public Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) database, was utilized in our study. The expression profiles of six samples consisting of 3 Fractured Day 2 α SMA-labeled cells (fractured samples) and 3 Unfractured Day 2 α SMA-labeled cells (unfractured samples, control samples) were used in this study. The platform for gene expression profile analysis was GPL6885 Illumina MouseRef-8 v2.0 expression beadchip (Illumina, San Diego, California, USA).

Preprocessing of the expression profile data

Two annotation files (GSE45156_family.soft and GSE45156_series_matrix.txt) were downloaded from the platform, and the normalized gene expression data were extracted. If more than one probe corresponded to one single gene, the averaged probe value was considered as the final expression value of the specific gene.

Differential analysis

Differentially expressed genes (DEGs) between fractured sample and control sample were selected using limma (Linear Models for Microarray Analysis, <http://www.bioconductor.org/packages/release/bioc/html/limma.html>) package in R.¹¹ The thresholds for DEG selection were false discovery rate (FDR) < 0.05 and $|\log_2(\text{fold change})| > 0.5$.

Function and pathway enrichment analysis

The Gene Ontology (GO, <http://www.geneontology.org/>) project provides ontologies to attributes of gene function in three domains, including molecular function, biological process and cellular component.¹² Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/pathway.html>) is a service of establishing manually curated pathway maps that contain current knowledge on biological networks.¹³ The DAVID (Database for Annotation, Visualization and Integration Discovery, <http://david.abcc.ncifcrf.gov/>) online tool¹⁴ was recruited to perform the GO and KEGG pathway enrichment analysis, to ascertain the functions

and pathways that might be disturbed by the identified DEGs, with the selection criterion of FDR < 0.1.

Construction of PPI network

Proteins often work together to exert a specific function during a biological process. To further explore the potential interactions among the DEGs from protein level, the STRTING (Search Tool for the Retrieval of Interacting Genes, <http://string-db.org/>) database¹⁵ was searched to screen the interplayed proteins of the protein production of the DEGs. The interactions with the confidence score > 0.7 that have been undergone experimental validation, text mining, co-expressed analysis and recorded in relevant databases were extracted to construct the PPI network, visualizing by Cytoscape software (<http://cytoscape.org/>).¹⁶ In the PPI network, a protein (the protein product of a DEG) serves as a node and the degree of a node reflects the number of the interplayed proteins with the specific node. Hub nodes were deemed as the nodes with high degrees.

Results

DEGs between fractured and control samples and their altered functions and pathways

Based on the aforementioned selection criteria, a subset of 960 DEGs (509 were up-regulated and 451 were down-regulated) were identified. As presented in Table 1, the up-regulated DEGs such as *Snrpd2* and *Eftud2* were significantly enriched in RNA splicing (GO: 0008380) and mRNA processing (GO: 0006397) BP, while *Plk1* and *Bub1b* were predominantly associated with cell cycle relating process (GO: 0007049, GO: 0022403 and GO: 0022402). Notably, *Snrpd2* and *Eftud2* were also significantly enriched in the pathway of Spliceosome (mmu03040) (Table 2).

On the other hand, the down-regulated DEGs such as *Ubc* were significantly enriched in skeletal system development (GO: 0001501) and bone development (GO: 0060348) BP (Table 1). Unfortunately, based on the pre-defined criteria, no significant pathways were enriched for the down-regulated DEGs.

PPI network of the identified DEGs

A PPI network of the DEGs was established, in which *Mrto4* (degree = 43), *Snrpd2* (degree = 35), *Eftud2* (degree = 38), *Bub1b* (degree = 36), *Plk1* (degree = 40), *Polr1a* (degree = 31), *Ubc* (degree = 79) and *Rps27a* (degree = 42) were the hub nodes (Fig. 1). Notably, *Snrpd2* was linked to *Eftud2*, whereas *Bub1b* was interacted with *Plk1*.

Discussion

Large amount of cellular activities are involved in the complex process of fracture healing.¹⁷ Through re-analyzing the expression profile data, GSE45156, we identified a set of 960 DEGs including 509 up-regulated genes and 451 down-regulated genes, which might be crucial for fracture healing. BPs such as RNA splicing and cell cycle were significantly enriched for the up-regulated DEGs such as *Snrpd2*, *Eftud2*, *Plk1* and *Bub1b*, whereas skeletal system development and bone development processes were the predominant ones for the down-regulated DEGs like *Ubc*. Notably, all the five genes were the remarkable nodes in the PPI network, in which *Snrpd2* was predicted to link to *Eftud2*, while *Bub1b* was to interact with *Plk1*.

RNA splicing, which is known as a crucial regulatory mechanism for gene expression, is a process mediated by large protein-RNA

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