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Research paper

Knockdown of EWSR1/FLI1 expression alters the transcriptome of Ewing sarcoma cells in vitro

Jihan Wang, Wenyan Jiang, Yuzhu Yan, Chu Chen, Yan Yu *, Biao Wang *, Heping Zhao *

Clinical Laboratory of Hong-Hui Hospital, Xi'an Jiaotong University College of Medicine, Xi'an 710054, China

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ABSTRACT

Ewing sarcoma breakpoint region 1 (EWSR1) fusion with Friend leukemia integration 1 transcription factor (FLI1) induced by a translocation of chromosome 11 with 22 contributes to Ewing sarcoma development. To date, the precise molecular mechanisms about EWSR1/FLI1 involving in Ewing sarcoma development remains to be defined. This study explored the potential critical gene targets of EWSR1/FLI1 knockdown in Ewing sarcoma cells on the gene expression profile based on online dataset, performed Limma algorithm for differentially expressed genes identification, constructed the transcriptional factor (TF)-gene regulatory network based on integrate transcriptional regulatory element database (TRED). The data showed up- and down-regulation of differentially expressed genes over time and peaked at 72 h after EWSR1/FLI1 knockdown in Ewing sarcoma cells. SMAD3 were up-regulated and FLI1, MYB, E2F1, ETS2, WT1 were down-regulated with more than half of their targets were down-regulated after EWSR1/FLI1 knockdown. The Gene Ontology (GO) and pathway annotation of these differentially expressed genes showed a consistent trend in each group of samples. Totally, there were 355 differentially expressed genes occurring in all five comparison groups of different time points, in which 39 genes constructed a dysregulated TF-gene network in Ewing sarcoma cell line A673 after EWSR1/FLI1 knockdown. These data demonstrated that knockdown of EWSR1/FLI1 expression led to transcriptome changes in Ewing sarcoma cells and that Ewing sarcoma development and progression caused by altered EWSR1/FLI1 expression may be associated with more complex transcriptome changes.

1. Introduction

Ewing sarcoma is a rare malignancy arising in the bone and soft-tissue and most frequently occurs in children and adolescence. Molecularly, Ewing sarcoma is characterized by a translocation between chromosome 11 and 22, leading to a fusion protein of Ewing sarcoma breakpoint region 1 (EWSR1) with Friend leukemia integration 1 transcription factor (FLI1). The latter is a transcriptional activator and responsible for up to 95% of Ewing sarcoma [1,2], although other chromosome translocations may also occur in Ewing sarcoma [3,4]. The wild-type EWSR1 is a member of ten-eleven translocation methylcytosine dioxygenase gene family and could interact with the general transcriptional components, like RNA polymerase II enzyme and TFIID complex to regulate gene expression [5,6]. FLI1 is a member of the ETS transcription factor family and functions as an oncogene to induce cell proliferation [7]. The EWSR1/FLI1 fusion is critical for Ewing sarcoma tumorigenesis. For example, cells expressing EWSR1/FLI1 fusion protein can form tumors after transplanting into immunodeficient mice [8,9], whereas cells expressing EWSR1 or FLI1 mutated protein lose the ability to form tumors in nude mice [10]. The EWSR1/FLI1 fusion protein is also important for cell growth regulation and gene expressions in other cell lines, including CTR rhabdomyosarcoma cells or RAT-1 fibroblasts [11,12]. However, to date, the precise molecular mechanisms about EWSR1/FLI1 fusion protein involving in Ewing sarcoma development remains to be defined; for example, how the EWSR1/FLI1 fusion protein interrupt normal cell cycle and why the EWSR1/FLI1 fusion protein only causes Ewing sarcoma or related tumors.

To this end, we proposed this study by analyzing the whole transcriptome after knockdown of EWSR1/FLI1 expression in Ewing sarcoma cell line A673. As we know, transcriptome instability always associated with cancer development [13], which should also be in Ewing sarcoma. Some key transcription factors (TF) may function as a tumor promoter or inhibitor in cells to regulate expression of their target genes. Thus, to better understand the transcriptional status affected by EWSR1/FLI1 protein in Ewing sarcoma, we first retrieved datasets from online database [14] and then analyze the data of transcriptome changes in Ewing sarcoma

* Corresponding authors.
E-mail addresses: 732867304@qq.com (Y. Yu), 527982150@qq.com (B. Wang), redcrossjyk@163.com (H. Zhao).

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cells at different periods of time points after knockdown of EWSR1/FLI1 and identifying the key TF and target genes. This study expects to provide insightful information regarding transcriptome alteration caused by EWSR1/FLI1 fusion protein and to identify the key TFs and targeting genes for future development of novel strategies in control of Ewing sarcoma.

2. Materials and methods

2.1. Retrieval of gene expression dataset from online database

In this study, we first downloaded the gene expression data series GSE27524 from GEO Datasets of NCBI (http://www.ncbi.nlm.nih.gov/gds/). The project GSE27524 provided a systematical analysis of gene expression using a cDNA microarray of A673 Ewing sarcoma cell line after inducible EWSR1/FLI1 knockdown with up to 96 h data [14]. The raw data GSE27524 were obtained from HG-U133A_2 platform (Affymetrix Human Genome U133A 2.0 Array) and all the microarrays raw data (.CEL) from this project were included for this study, which contains 4 samples of 0 h, 3 samples of 18 h, 3 samples of 36 h, 2 samples of 54 h, 2 samples of 72 h, 2 samples of 96 h after EWSR1/FLI1 knockdown in A673 Ewing sarcoma cell line. After downloading the raw data, we applied R statistics analysis language and software for data processing. First we have to check the quality of the data, results showed a qualified quality and reasonable sample cluster of all the 16 microarray raw datasets (Supplementary Fig. 1).

2.2. Profiling of differentially expressed genes (DEGs) in Ewing sarcoma after EWSR1/FLI1 knockdown

To identify DEGs in Ewing sarcoma after EWSR1/FLI1 knockdown, we utilized the limma algorithm package in R/Bioconductor to identify differentially expressed genes between two groups at a time [15,16]. The dataset on 0 h was used as the control group and we compared the other five time points after EWSR1/FLI1 knockdown to the 0 h control. Genes with p-value < 0.05, false discovery rata (FDR) < 0.05 and fold change (FC) > 2 between two groups were considered as the DEGs.

2.3. Analysis of the TF-gene regulatory network based on gene expression and transcriptional regulatory element database (TRED)

The TRED (http://rulai.cshl.edu/TRED) provides an accurate and comprehensive knowledge involves transcriptional regulatory elements [16,17]. Thus, we first obtained all the transcription factor-gene regulation modes on 36 cancer-related TF families from TRED, then constructed the dysregulated TF-genes regulatory network by integrating DEGs with transcriptional regulation modes. The Cytoscape software was utilized to visualize such regulatory networks [16,18]. For example, triangles in the network are TFs (orange for up-regulated, yellow for down-regulated), circles in the network are target genes (red for up-regulated and green for down-regulated) in A673 Ewing sarcoma cells after EWSR1/FLI1 knockdown. Relationship between TFs and their targets were interacted by arrows, direction of the arrow was from the Source (TFs) to the Target (genes).

2.4. Functional annotations of DEGs

To explore the function of selected DEGs, we applied the online analytical tools [Database for Annotation, Visualization and Integrated Discovery (DAVID)] to obtain the Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment [19]. We first uploaded the selected DEGs (OFFICIAL_GENE_SYMBOL) list into DAVID tools for functional annotation, and then collected data information from "GENE_ONTOLOGY" [including GO_BP (Gene Ontology_Biological Process), GO_CC (Gene Ontology_Cellular Component) and GO_MF (Gene Ontology_Molecular Function)] and "KEGG_PATHWAY" for GO and pathway analysis. GO and KEGG terms with $P < 0.05$ were selected as statistically significant enriched.

3. Results

3.1. Identification of differentially expressed genes (DEGs) in Ewing sarcoma after EWSR1/FLI1 knockdown

In this study, we first identified DEGs in Ewing sarcoma after EWSR1/FLI1 knockdown by using the downloaded gene expression datasets GSE27524 from GEO Datasets of NCBI (http://www.ncbi.nlm.nih.gov/gds/). The datasets GSE27524 contained a cDNA microarray analysis of A673 in Ewing sarcoma cell line after inducible EWSR1/FLI1 knockdown with up to 96 h data [14]. We applied the limma algorithm in R/Bioconductor for genes with a p-value < 0.05 and fold change > 2 to considered as DEGs compared to the 0 h control. Fig. 1 shows the number of DEGs in the five comparison groups, detail informations were summarized in Supplementary Table 1. Results indicated that fewer DEGs occurred in 18 h time point vs. 0 h time point and the DEGs reached the maximal numbers in 72 h time point after EWSR1/FLI1 knockdown (Fig. 1).

3.2. Functional annotations of selected DEGs

We then uploaded these DEGs in all five groups to the DAVID tool for functional annotation. Fig. 2 and Supplementary_GO_KEGG showed the number and detailed information of GO-BP, GO-CC, GO-MF and KEGG pathway terms that DEGs involved in each group. We found that the changes of GO and KEGG terms showed almost the consistent trend with DEGs changes in each time point, that peaked in 72 h. We then focused on the DEGs that aberrantly expressed in all five groups to explore the biology function changes in Ewing sarcoma cells after EWSR1/FLI1 knockdown. A total of 355 genes were obtained, including 172 upregulated DEGs and 180 downregulated DEGs in all of these five comparison groups, while three DEGs showed a different

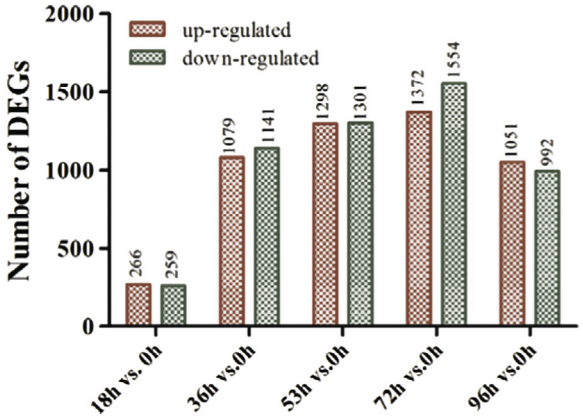

Fig. 1. Differentially expressed genes after EWSR1/FLI1 knockdown in Ewing sarcoma cell**s.** The gene expression datasets GSE27524 were downloaded from GEO Datasets of NCBI (http://www.ncbi.nlm.nih.gov/gds/). The datasets GSE27524 contained a cDNA microarray analysis of A673 in Ewing sarcoma cell line after inducible EWSR1/FLI1 knockdown with up to 96 h data [14]. We performed the limma algorithm in R/Bioconductor for genes with a p-value < 0.05 and fold change > 2 to considered as DEGs compared to the 0 h control.