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 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.

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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 target 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 rate (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
expression pattern among these five groups (Supplementary Table 2). The biological function and pathway annotation using the DAVID tool indicated that the upregulated DEGs involved in 102 GO-BP, 16 GO-CC, 23 GO-MF and 7 KEGG terms, the downregulated DEGs involved in 42 GO-BP, 21 GO-CC, 9 GO-MF and 2 KEGG terms. Table 1 and 2 summarize the KEGG terms and top 3 significant GO terms (including GO-BP/CC/MF) of DEGs.

### 3.3. Changes in transcription factors in Ewing sarcoma cells after EWSR1/FLI1 knockdown

To explore the transcriptome changes after EWSR1/FLI1 knockdown in Ewing sarcoma cells, we utilized the TRED database to perform transcriptome analysis. The TRED database provides comprehensive and accurate trans-regulatory information for target genes of more than 140 cancer-related transcription factors (belong to 36 cancer-related TF families). Our data summarized in Table 3 show the differentially expressed TFs in these five comparison groups. Besides FLI1 knockdown, other eight TFs also showed changes in all five groups, including TFAP2B/SMAD3 up-regulation and WT1/EGR4/ETS2/E2F1/E2F5/MYB down-regulation. The trend of the aberrant TFs was in accordance with DEGs for each comparison group and peaked in 72 h after EWSR1/FLI1 knockdown. Furthermore, based on the trans-regulatory information from this transcriptional regulatory element database, we further identified the dysregulated TF-genes network involved in these 355 genes that were aberrantly expressed in all five groups. This network containing six TFs and 33 targets that including 42 different regulation modes (Fig. 3A). More than half of the genes in this network were down-regulated (including transcription factors MYB/ETS2/FLI1/E2F1/WT1 and 18 targets) and Fig. 3B shows the bi-clusters analysis data on these 39 genes (containing six TFs) in

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**Table 1.**

| KEGG term                    | P    | Fold enrichment | Gene names                        |
|------------------------------|------|-----------------|-----------------------------------|
| **Upregulated genes**        |      |                 |                                   |
| ECM-receptor interaction     | < 0.01 | 6.52           | ITGA6, COL6A3, COL3A1, COL1A2, COL6A1, ITGA4, FN1 |
| Focal adhesion               | < 0.01 | 3.89           | EGFR, ITGA6, COL6A3, COL3A1, COL1A2, PDGFRα, COL6A1, ITGA4, MYLK, FN1 |
| Axon guidance                | < 0.01 | 4.85           | SEMA6A, PLXNC1, UNC5B, GNA1, L1CAM, SEMA3A, UNC5C, CXCL12 |
| Pathways in cancer           | 0.02  | 2.39           | EGFR, PLD1, CBLB, PTG52, ITGA6, PDGFRα, SMAD3, FOXD1, TCF7L2, FN1 |
| Calcium signaling pathway    | 0.02  | 3.11           | EGFR, ATP2B1, ATP2B4, PLCB4, PDGFRα, ITPR1, MYLK |
| Prostate cancer              | 0.03  | 4.39           | EGFR, PDGFRα, CREB3L1, FOXD1, TCF7L2 |
| Gap junction                 | 0.03  | 4.39           | EGFR, PLCB4, GNA1, PDGFRα, ITPR1 |
| **Downregulated genes**      |      |                 |                                   |
| Cell cycle                   | < 0.01 | 6.21           | E2F1, CDC5, CCND1, CDC45, E2F5, SKP2, PKMYT1, MCM4, CDC25A |
| Purine metabolism            | 0.03  | 3.38           | POLR3G, POLR3K, RRM2, PDE4A, PDE3B, GART |

P: modified fisher exact p-value. The smaller, the more enriched.
Table 2.
Top 3 significant GO of DEGs in Ewing sarcoma cells after EWSR1/FLI1 knockdown.

| GO term                                      | P    | Fold enrichment | Gene names                                                                 |
|----------------------------------------------|------|-----------------|----------------------------------------------------------------------------|
| **Upregulated genes**                        |      |                 |                                                                            |
| (BP) cell morphogenesis involved in differentiation | < 0.01 | 6.03            | L1CAM, SOX9, CXCL12, SEMA6A, UNC5B, ANK3, TGFBR3, SEMA3A, UNC5C, APBB2, NEFL, DST, GAP43, DCLK1, FN1 |
| neuron projection morphogenesis              | < 0.01 | 6.44            | EGFR, L1CAM, CXCL12, GAS7, SEMA6A, UNC5B, ANK3, SEMA3A, UNC5C, APBB2, NEFL, DST, DCLK1, GAP43 |
| cell morphogenesis                           | < 0.01 | 4.68            | EGFR, L1CAM, SOX9, CXCL12, GAS7, SEMA6A, UNC5B, ANK3, TGFBR3, SEMA3A, UNC5C, APBB2, NEFL, DST, GAP43, DCLK1, FN1 |
| (CC) extracellular region part               | < 0.01 | 2.32            | EGFR, SPARC1, SOR1, COL3A1, OLIML2A, NID2, CXCL12, VCA1, DK1, BGN, TGFBI, COL6A3, COL1A2, TGFBR3, COL6A1, STC1, ANGPT1, MAFAP4, DST, BMP5, FN1, IGFBP5, VLDLR |
| extracellular matrix                         | < 0.01 | 6.62            | COL6A3, COL3A1, COL1A2, COL6A1, NID2, MFAP4, DST, FN1                       |
| (MF) calmodulin binding                      | < 0.01 | 6.52            | ATP2B1, ATP2B4, CALD1, MARCKS, GEM, RGS16, ADD3, GAP43, MYLK                |
| platelet-derived growth factor binding       | < 0.01 | 36.88           | COL3A1, COL1A2, PDGFRA, COL6A1                                             |
| integrin binding                             | < 0.01 | 10.31           | VCAM1, ITGA6, TGFBI, COL3A1, L1CAM, DST                                   |
| DNA replication                              | < 0.01 | 6.17            | CDC6, DTL, GINS3, NASP, MCM10, MCM4, C100RF2, CDC25A, RFC3, CDC45, DK1, RRM2, CHAF1A |
| DNA metabolic process                        | < 0.01 | 3.74            | EXO1, CDC6, XRC1C1, DTL, UNC, NASP, GINS3, BRIP1, MCM10, MCM4, CDC25A, C100RF2, RFC3, CDC45, DCLRE1A, DCLRE1B, DED1, RRM2, BCL11B, CHAF1A, ADRA1D |
| ribosome biogenesis                          | < 0.01 | 8.13            | EXOS9, DCK1, NOLC1, EXOSC7, NIP7, BYS, BRI1, RSI1, RPL5, RRP9, NOP56       |
| (CC) intracellular organelle lumen           | < 0.01 | 2.94            | E2F1, GRPEL1, E2F5, PKMYT1, TIMM50, MCM10, CDC45, DK3, DDX11, URB2, AEN, TARDBP, MYB, CDC6, EXOSC9, EXOS7, NIP7, BYS, RRP9, MCM4, C100RF2, CD3EAP, RFC3, CNDC1, ALDH1B1, MYBBP1A, NUFIP1, ZBTB16, WTI, SRTF, PPAN, NUF50, TEAD4, BRI1, RPL5, NKX2–2, POLR3G, POLR3K, POI1, CDC25A, IDHA3, PPIF, PKL4, NOLC1, NOP16, POP1, RRS1, NOP56, UTP20, DDX52 |
| nuclear lumen                                | < 0.01 | 3.25            | E2F1, E2F5, NUF1P1, PKMYT1, TIMM50, MCM16, MCM10, WTI, PPAN, SRTF, CDC45, DK1, DDX11, URB2, AEN, TARDBP, BRI1X, NOP50, TEAD4, RPL5, MYB, MYBBP1A, POLR3G, CDC6, EXOSC9, POLR3K, EXOS7, NIP7, BYS, RNP1, RRS1, NOP56, UMF50, UTP20, DDX52, NKX2–2 |
| organelle lumen                              | < 0.01 | 2.89            | E2F1, GRPEL1, E2F5, PKMYT1, TIMM50, MCM10, CDC45, DK3, DDX11, URB2, AEN, TARDBP, MYB, CDC6, EXOSC9, EXOS7, NIP7, BYS, RRP9, MCM4, C100RF2, CD3EAP, RFC3, CNDC1, BDNL1, NOP16, POP1, RRS1, NOP56, UTP20, DDX52, NKX2–2 |
| (MF) RNA binding                             | < 0.01 | 2.26            | EXOS9, PUS1, EXOS7, NIP7, PO1, NUF1P1, TIMM50, RPP9, RR0B1, WTI, DCK1, DDX11, TARDBP, RPL5, NOP56, AKA1, DDX52 |
| structure-specific DNA binding               | < 0.01 | 4.61            | EXO1, ZBTB16, ROB1, MCM4, WTI, C100RF2, FEV                              |
| DNA helicase activity                        | 0.01  | 9.55            | DX11, BRP1, MCM4, C100RF2                                                |

*P* modified fisher exact *p*-value. The smaller, the more enriched.
the network (refer to Supplementary Fig. 2).

4. Discussion

Translocation of chromosome 11 with 22 causes Ewing sarcoma breakpoint region 1 (EWSR1) fusion with Friend leukemia integration 1 transcription factor (FLI1) and in turn contributes to up to 95% of Ewing sarcoma. However, to date, the underlying molecular mechanisms responsible for EWSR1/FLI1 expression-induced Ewing sarcoma development remain to be defined. Thus, the current study explored the transcriptome changes after EWSR1/FLI1 knockdown in Ewing sarcoma cells. We utilized a combination of gene expression array data to perform the limma algorithm in R/Bioconductor to obtain DEGs and then DAVID annotation and transcriptional regulatory element database to identify transcriptome changes after EWSR1/FLI1 knockdown in Ewing sarcoma cells. After assessed the aberrantly expressed genes at different time points after EWSR1/FLI1 knockdown, we found an increasing DEGs over time and peaked in 72 h, which is almost consistent with GO-BP/CC/MF and KEGG pathway annotation changes over time, suggesting that EWSR1/FLI1 expression altered the transcriptome instability and subsequent biological alterations in Ewing sarcoma.

Specifically, we found 355 DEGs in all of the five comparison groups vs. the 0 h control after EWSR1/FLI1 knockdown, although the number of DEGs in each group was different. Expression patterns of these 355 DEGs (except three genes) were almost the same in these five comparison groups, including 172 upregulated DEGs and 180 downregulated DEGs, which involved in nine significant gene pathways. For example, the ECM-receptor interactions and the focal adhesion are the key pathways involved in cancer metastasis [20]. The EWS/FLI oncogene was reported to drive changes in cellular morphology, adhesion and migration of Ewing sarcoma. Moreover, these DEGs were also enriched in the axon guidance and calcium signaling pathways. No direct association of axon guidance pathway with Ewing sarcoma has ever been reported yet. In a research of osteosarcoma, the differentially expressed genes in metastatic osteosarcoma was enriched in the axon guidance and focal adhesion according to biological process. The most significant gene pathway for the downregulated DEGs involved in regulation of the cell cycle. Researches had showed

Table 3. Differentially expressed TFs among these five time-points vs. 0 h control in Ewing sarcoma cells after EWSR1/FLI1 knockdown.

| Time (h) | Upregulated TFs | Downregulated TFs |
|----------|----------------|-------------------|
| 18 h     | TFAP2B, SMAD3, PAX6 | WT1, EGR4, MYC, ETS2, E2F1, E2F2, E2F5, MYB, FLI1 |
| 36 h     | JUNB, TFAP2A, TFAP2B, TFAP2C, ATF3, EGR2, EGR3, GLI1, PPARD, SMAD3, STAT6 | BRCA1, BRCA2, E2F1, E2F3, E2F4, EGR4, ETS2, FLI1, MYB, MYBL1, MYBL2, MYC, STAT1, WT1 |
| 53 h     | FOX, FOSB, JUN, JUNB, TFAP2A, TFAP2B, TFAP2C, ATF3, ATF4, ATFB, BCL3, CEBPD, CREB5, EGR1, EGR2, EGR3, GLI3, EPAS1, MSH1, NFkB1, PPARD, RAR, SMAD2, SMAD3, SMAD5, STAT3, STAT6 | BRCA1, BRCA2, E2F1, E2F3, E2F5, EGR4, ELK4, ETS2, FLI1, MYB, MYBL1, MYBL2, POU3F1, WT1 |
| 72 h     | EPAS1, TFAP2C, RARB, TFAP2B, SMAD3, JUNB, BCL3, CEBPD, SMAD7, ELK3, BCL6, PPARD | PAX2, POU3F1, ELK4, PAX6, EGR4, E2F1, WT1, ETS2, MYB, MYBL1, E2F5, BRCA1, FLI1, MYB, BRCA2, E2F1, MYBL2 |
| 96 h     | EPAS1, TFAP2C, RARB, TFAP2B, SMAD3, JUNB, BCL3, CEBPD, SMAD7, ELK3, BCL6, PPARD | WT1, BRCA1, POU3F1, SMAD6, ELK4, MYC, EGR4, PAX9, BRCA2, MYBL2, E2F1, MYB, ETS2, E2F5, FLI1 |

The underlines were differentially expressed TFs that occurred in all five time-points.

Fig. 3. Construction of the TF-gene regulatory network and bi-clusters analysis of these DEGs. (A) The regulation information are derived from TRED. Triangles are TFs (orange for up-regulated, yellow for down-regulated); circles are target genes (red for up-regulated genes and green circles for down-regulated genes). The direction of arrows is from the Source (TFs) to the Target. (B) The Bi-cluster analysis of all DEGs in the TF-gene regulatory network based on gene expression value in each sample. Each row represents a gene and each column represents a sample.
that genes transcriptionally deregulated by EWSR1/FLI1 have the potential to contribute to malignant transformation, such as cell cycle regulators cyclinD1, indicated that EWSR1/FLI1 knockdown may partly inhibit the cell cycle progression of Ewing sarcoma [21]. In addition to cancer-related gene pathways, the down-regulated DEGs were also enriched in purine metabolism, revealed that EWSR1/FLI1 may involved in Ewing sarcoma tumorigenesis and progression by upregulation of purine metabolism for DNA synthesis and cell proliferation. These data indicate that EWSR1/FLI1 fusion protein alters cell cycle and upregulates purine and pyrimidine metabolism. Future study will focus on how and whether to manipulate these gene pathways for reverse Ewing sarcoma tumorigenesis.

Furthermore, our current study also explored the transcriptome changes caused by EWSR1/FLI1 knockdown in Ewing sarcoma and found that there were nine transcription factors dysregulated in all of these five comparison groups compared to control after EWSR1/FLI1 knockdown. Six of these nine pathways could construct a TF-gene regulatory network. As the only upregulated TF in this network, expression of SMAD3 and three of its targets were increased by the proteasome inhibitor in Ewing sarcoma cells and B-MYB accumulation could promote tumor progression [22]. Recently, EWS-FLI1 fusion protein showed to function as an E2F switch to drive target gene expression, activation of E2F3 protein cooperated with ETS on the targeting gene promoters could consequently lead to an aberrant cell cycle activation [23]. In addition, WT1 protein is able to regulate target gene transcription and miRNA splicing to contribute to angiogenesis in Ewing sarcoma by increase in expression of pro-angiogenic molecules (VEGF, MMP9, Ang-1 and Tie-2) [24].

In conclusion, the current study explored altered transcriptome after EWSR1/FLI1 knockdown in Ewing sarcoma cells and identified a dysregulated TF-gene regulatory network. However, our current study is just proof-of-principle and further researches are needed to experimentally manipulate the gene pathways after EWSR1/FLI1 knockdown or knock-in in Ewing sarcoma cells to confirm their role in mediation of EWSR1/FLI1 fusion protein in development of Ewing sarcoma.

Conflict of interest statement

The authors declared that there is no conflict of interest in this work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jbo.2016.05.006.

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