Molecular profiling of TOX-deficient neoplastic cells in cutaneous T cell lymphoma

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Abstract

Cutaneous T cell lymphoma (CTCL) is a rare but potentially devastating primary cutaneous lymphoma. CTCL is characterized by localization of neoplastic T lymphocytes to the skin, with mycosis fungoides (MF) and its leukemic form, Sézary syndrome (SS) being the most common variants. Thymocyte selection-associated high-mobility group box (TOX) gene has been found to be highly expressed in MF and SS. It is reported that higher expression levels of TOX in patients will increase risks of disease progression and poor prognosis. However, the molecular events leading to these abnormalities have not been well understood. To better understand the molecular mechanism underlying TOX-mediated differentially expressed genes (DEGs) in CTCL, and to identify DEGs pathways triggered after knockdown of TOX gene in the CTCL cell line Hut78, we employed two shRNA-mediated lentiviruses to knock down TOX gene in the skin lymphoma cell line HuT78. RNA sequencing (RNAseq) analysis was applied to analyze DEGs, DEGs GO and their corresponding pathways. Knockdown of TOX can induce upregulation of 547 genes and downregulation of 649 genes, respectively. HOXC9 was the most significant downregulated gene. Most DEGs are enriched in malignancies and relate to the Wnt and mTOR signaling pathways, and therefore they can regulate cellular processes and induce different biological regulation. Transcriptome analysis of DEGs after knockdown of TOX in our study provides insights into the mechanism of TOX in CTCL and suggests candidate targets for therapy of CTCL.

Keywords Cutaneous T cell lymphoma · TOX · RNA sequencing analysis · Differentially expressed gene · Signaling pathway

Introduction

CTCLs are a heterogeneous group of non-Hodgkin lymphoproliferative disorders characterized by accumulation and expansion of neoplastic T lymphocytes to the skin [28]. MF and SS constitute two main subtypes of CTCL. While MF primarily affects the skin, SS is characterized by the presence of circulating malignant Sézary cells. Together, MF and SS account for 65–80% of CTCL cases [9, 11, 23]. Although accumulative evidence indicates that defects in apoptosis and cell cycle control are critical in disease pathogenesis [5, 21], the molecular mechanism leading to these abnormalities has not been well understood yet.

The TOX gene was firstly described in 2002 [27], as a part of the superfamily of high-mobility group box proteins that act as regulators of gene expression, mainly by modifying the chromatin structure [10, 27]. TOX mRNA is most abundant in the thymus, liver and brain [27]. TOX is
involved in lymphocyte maturation, and Zhang et al. demonstrated that TOX was highly and specially expressed in early MF [31]. After this, several studies have confirmed that TOX is aberrantly expressed in CD4+/CD8⁻ neoplastic T cells in MF and SS [2, 6, 7, 15, 19, 20, 29], so as to be aberrantly expressed in CTCL with CD4⁻/CD8⁺ and CD4⁻/CD8⁻ phenotypes [24], differentiating malignant from non-malignant skin-infiltrating T cells found in benign inflammatory dermatoses [31]. Aberrant expression of TOX plays a central role in malignant survival, proliferation, and tumor formation in CTCL [7]. Stable knockdown of TOX in CTCL cells has promoted apoptosis and reduced cell cycle progression, leading to less cell viability and colony-forming ability in vitro and reducing tumor growth in vivo [7].

It is generally believed that abnormal gene expression is a key process in disease initiation and progression. Hut78 cell line derived from SS exhibits high expression of TOX, and TOX-deficient Hut78 cells can promote apoptosis and reduce cell cycle [7], but its mechanism is not very clear. Herein, we applied RNAseq analysis to further explore transcriptional changes including expressed genes (DEGs), DEG Gene Ontology (GO) and pathways in TOX-deficient Hut78 cells.

Material and methods

**Cell culture**

Human CTCL cell line Hut78 (ATCC no. TIB161) was cultured in RPMI 1640 and 10%FBS as described by the American Type Culture Collection (Manassas, VA). Infected CTCL cells were cultured in the above medium plus puromycin.

**Lentivirus infection**

Lentivirus vector (hU6-MCS-Ubiquitin-EGFP-IRESPuromycin) and shRNA sequence were designed and synthesized by Genechem (Shanghai, China). Destination cells were infected with lentiviral supernatants, using 8 µg/ml polybrene and high virus titer for MOI ≥ 100. After 48–72 h of incubation, the supernatant was replaced by a medium containing 1 mg/ml puromycin.

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from cell pellets using TRIzol (Invitrogen, Thermo Fisher) according to the manufacturer’s protocol. cDNA synthesis was performed using the GoScript™ Reverse Transcription System Kit (A5000) from Promega. qPCR reactions were performed with FastStart Universal SYBR Green Master (Rox) from Roche. The experiments were performed according to the manufacturer’s instructions. The sequences of the primers used for qRT-PCR analyses are listed in Table S3. All reactions were run in triplicate. The CT values were calculated using the standard curve method.

**Western blotting**

After lentivirus infection, HuT78 cell pellets were prepared by centrifugation at 300g, and then total cells were lysed in RIPA buffer (50 mM Tris, 150 mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% sodium deoxycholate). After removing insoluble material by centrifugation at 10,000g at 4 °C for 5 min, total protein concentration was determined using BCA assay as per manufacturer’s instruction with a microplate reader. 40 µg protein was used for SDS-PAGE gel electrophoresis (Bio-Rad) and transferred onto PVDF membranes (Bio-Rad). Blocking was done with 5% milk and then the membranes were incubated with primary antibodies, anti-TOX (1:1000 HPA018322, Sigma-Aldrich) or anti-actin (1:5000, A1978, Sigma-Aldrich) overnight at 4 °C. After washing, membranes were incubated with secondary antibodies (peroxidase-conjugated, suitable for each primary antibody) for 2 h at room temperature. The signal was detected using Bio-Rad ChemiDoc XRS + System after adding Super Signal West Pico chemiluminescence.

**Apoptosis detection**

The treated Hut78 cells (1 × 10⁶) using shRNA 1 construct were transferred to a 15 ml centrifuge tube. Annexin V binding buffer was added. After centrifugation at 2000 rpm for 5 min at 4 °C, the cells were washed three times and 100 µl of binding buffer, 5 µl of Annexin V-APC and 10 µl of 7-AAD stain (Thermo Fisher Scientific, Inc) were added and incubated in the dark for 25 min. Detection of apoptotic cells was performed by flow cytometry.

**Cell cycle analysis**

The treated Hut78 cells (1 × 10⁶) using shRNA 1 construct were collected and fixed with 75% ice-cold ethanol at 4 °C overnight and then stained with 5 µl propidium iodide (Thermo Fisher Scientific, Inc.) at room temperature for 5 min in the dark. The cell cycle distribution was analyzed by flow cytometry.

**RNAseq analysis**

Total RNA from infected cells was harvested and extracted by using TRIzol (Invitrogen, Thermo Fisher). Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) was used to
to knockdown TOX as shown in Fig. 1d. cells in G0/G1 phase and less cells in G2/M phase after
employed to analyze cell apoptosis, and we observed that
also diminished as shown in Fig. 1b with the sh1 group
mRNA expression (p < 0.05). TOX protein expression was
downregulated in TOX-deficient Hut78 cells.

Results

Genetic silencing of Tox in Hut78 cells

To investigate the transcriptional changes after TOX knock-
down, two lentivirus targets were designed to knock down
TOX gene in Hut78 cell line, as presented in Table S2. After
lentivirus infection, RT-qPCR and Western blot were com-
pleted. TOX expression was significantly reduced in mRNA
level as shown in Fig. 1a: Compared to the NC group, both
sh1 and sh2 groups demonstrate significantly reduced TOX
mRNA expression (p < 0.05). TOX protein expression was
also diminished as shown in Fig. 1b with the sh1 group
showing more inhibition of TOX expression than the sh2
group. Annexin V-APC/7AAD flow cytometry assay was
employed to analyze cell apoptosis, and we observed that
apoptotic cells were increased after knockdown of TOX as
shown in Fig. 1c. The cell cycle distribution analysis showed
more cells in G0/G1 phase and less cells in G2/M phase after
knockdown of TOX as shown in Fig. 1d.

DEGs after TOX knockdown

After RNAseq and reads filtering, we mapped clean reads
to reference genome by using Bowtie 2 [12] and then calcu-
lated the gene expression level for each sample with RSEM
[13], a software package for estimating gene and isoform
expression levels from RNAseq data. Subsequently, we cal-
culated Pearson correlation between all samples by using
cor, performed hierarchical clustering between all samples
by using hclust, performed PCA analysis with all samples
using princomp, and drew the diagrams with ggplot2 with
functions of R. The number of genes and transcripts in each
sample are shown in Table1. We further calculated the heat
distribution of the gene expression level of each sample, so that we could observe the disper-
sion of the distribution (results as shown in Fig. S1b). Based
on the gene expression level, we could identify the DEGs
between samples or groups. MA plots were used to show
the distributions of DEGs in Fig. S1. Compared to the NC
group, 3897 genes were overexpressed and 2702 genes
were underexpressed in group sh1 (Fig. S1c). Compared to
the NC group, 2723 genes were overexpressed and 3224
genes were underexpressed in group sh2 (Fig. S1d). Taken
together, after TOX knockdown, a total of 547 genes were
upregulated and 649 genes were downregulated. The top
20 downregulated genes are listed in Table 2 and the top
20 upregulated genes are listed in Table S1. Interestingly,
we found that multiple genes in the HOX gene family were
downregulated in TOX-deficient Hut78 cells.

GO analysis of DEGs

With DEGs, we performed Gene Ontology (GO) classifica-
tion and functional enrichment. GO has three main ontolo-
gies: molecular biological function, cellular component and
biological process. The GO classification results are shown
in Fig. 2a, b. We used DAG (directed acyclic graph) to show
the GO enrichment result. Each bar shows GO terms, and the
amount of up- or down-regulated genes are shown in Fig. 2c,
d. In our study, we found that TOX gene knockdown could
significantly influence the cellular process, the cell growth
as well as the death signal transduction, as was previously
reported [7]. Among most of the enriched GO terms, most
DEGs were related to cellular process, biological regulation
and binding process.

Pathway analysis of DEGs

To examine the expression profile of DEGs in our result,
DEGs (both upregulated and downregulated) were then sub-
jected to the KEGG pathway enrichment analysis. More than
23% of the DEGs could be annotated. The pathway classifi-
cation results comparing group NC and group sh1/sh2 are
shown in Fig. 3a and supplementary Fig. S2a, and the path-
way functional enrichment results are shown in Fig. 3b and
supplementary Fig. S2b. The pathway functional enrichment
results for up- or down-regulated genes are shown in Fig. 3c
and supplementary Fig. S2c. The top ten KEGG pathways
with the highest representation of the DEGs are shown in
Table 3. We found that most DEGs were enriched in cancer
pathways (ko05200), including breast cancer (ko05224),
gastric cancer (ko05226) and hepatocellular carcinoma
(ko05225), and that some DEGs were also enriched in Wnt
(ko04310), mTOR (ko04150) signaling pathways and path-
ways in regulating pluripotency of stem cells (ko04550).
Lentivirus infection knockdown TOX gene expression. TOX knockdown by 2 shRNAs (sh1 and sh2, both specific for TOX mRNA) and negative control (a non-targeting shRNA). Infected cells were selected by puromycin (1 mg/mL) for 5 days. mRNA and protein were extracted for further analysis. 

Table 1

| Sample  | Total gene number | Known gene number | Novel gene number | Total transcript number | Known transcript number | Novel transcript number |
|---------|-------------------|-------------------|------------------|-------------------------|-------------------------|------------------------|
| NC_1    | 16,143            | 13,262            | 2881             | 28,211                  | 14,795                  | 13,416                 |
| NC_2    | 15,472            | 12,807            | 2665             | 25,537                  | 13,585                  | 11,952                 |
| NC_3    | 15,468            | 12,863            | 2605             | 25,801                  | 13,699                  | 12,102                 |
| sh1_1   | 15,089            | 12,530            | 2559             | 24,071                  | 12,629                  | 11,442                 |
| sh1_2   | 14,639            | 12,088            | 2551             | 21,614                  | 11,028                  | 10,586                 |
| sh1_3   | 15,023            | 12,387            | 2636             | 23,159                  | 11,918                  | 11,241                 |
| sh2_1   | 16,040            | 13,100            | 2940             | 28,513                  | 15,031                  | 13,482                 |
| sh2_2   | 16,281            | 13,395            | 2886             | 30,287                  | 16,217                  | 14,070                 |
| sh2_3   | 13,278            | 11,548            | 1730             | 20,495                  | 11,131                  | 9364                   |

Discussion

TOX is aberrantly overexpressed in CTCLs, such as MF and SS. Stable knockdown of TOX in CTCL cells reduces cell cycle progression and promotes apoptosis, leading to inhibited cell viability and colony-forming ability in vitro and suppressed tumor growth in vivo [12]. After TOX gene knockdown, many genes are highly expressed, such as two cyclin-dependent kinase inhibitors (CDKNs), including CDKN1B and CDKN1C) [15]. It has been reported that TOX is able to regulate cell cycle in primary Sézary cells and cutaneous T cell lymphoma, whereas TOX knockdown leads to cell cycle arrest and secondary cell death [12, 16]. In our study, we found that, after TOX knockdown, some proliferation and apoptosis-associated genes, such as PFKFB3, CDK5 and CKKN2A, were up- or down-regulated and most DEGs were enriched in cellular process and cancer pathways, which highlights the importance of TOX in cancer process. As we noted, both changes in apoptosis and cell cycle characteristics of the groups after gene knockdown, it is not clear if the differentially expressed genes are directly the result of interactions with TOX or the result of downstream cell cycle-dependent changes.

HOX genes, including HOX C9, HOX C4, HOX C5, HOX C8, HOX C10, HOX C11 and HOX C13, were significantly downregulated after TOX knockdown, with HOX C9 being downregulated to the highest degree. HOX genes are homeobox genes that function as transcription factors. In humans, 39 HOX genes have been assigned to 13 paralogous groups in four separate clusters termed HOXA, HOXB, HOXC and HOXD [8]. HOX C9 is aberrantly expressed in breast cancer, lung cancer, body fat mass and astrocytoma [3, 8, 14, 22]. HOX C9 can induce neuronal differentiation of neuroblastoma cells [26]. Wang et al. [25] demonstrate that HOX C9 can directly regulate distinct sets of genes to coordinate diverse cellular processes during neuronal differentiation. This may explain why TOX knockdown will lead to less cell viability and colony-forming ability in vitro and reduce tumor growth in vivo.

Through DEGs GO analysis, we found most DEGs are related to the cellular process, biological regulation and binding process. This can explain why TOX knockdown will induce inhibited cell viability as previously reported [7]. With DEGs pathway analysis applied in KEGG, we find two important tumor-related pathways, Wnt and mTOR. They are generally associated with cellular proliferation, differentiation and apoptosis in invertebrates and mammals [4]. β-catenin is expressed by tumor cells in cutaneous lymphoproliferative disorders at various frequencies, and activation and accumulation of β-catenin plays an important role in the development of skin lymphomas [1]. CTCL cells display mTORC1 activation in the lymphoma stage-related fashion with the highest percentage of positive cells identified at the late stage [17]. Treatment with rapamycin can persistently...
Table 2  Top 20 significantly downregulated genes after TOX knockdown

| Gene name | GeneID | Length | NC-expression | sh1-expression | log2 Ratio (sh1/NC) | q value | p value | sh2-expression | log2 Ratio (sh2/NC) | q value | p value |
|-----------|--------|--------|---------------|----------------|---------------------|---------|---------|---------------|---------------------|---------|---------|
| HOXC9     | 3225   | 1528   | 90,574        | 16,021         | −1.837919802        | 0.0     | 0.0     | 16,747        | −2.788427035        | 0.0     | 0.0     |
| PIGS      | 94005  | 2643   | 54,217        | 7133           | −2.264951439        | 0.0     | 0.0     | 9093          | −2.929149771        | 0.0     | 0.0     |
| TXNDC16   | 57544  | 2854   | 3017          | 68             | −4.810223171        | 0.0     | 0.0     | 313           | −3.622112876        | 0.0     | 0.0     |
| GPATCH4   | 54865  | 2158   | 8047          | 2539           | −1.002977669        | 6.4747E-244 | 7.0734E-246 | 4285          | −1.26238857         | 0.0     | 0.0     |
| CKB       | 1152   | 1475   | 3529          | 984            | −1.18131624         | 6.1571E-140 | 1.7733E-141 | 907           | −2.31331716         | 0.0     | 0.0     |
| UNC119B   | 84747  | 4423   | 3865          | 1210           | −1.014248397        | 7.3431E-120 | 2.5525E-121 | 2313          | −1.09393589         | 4.2E-189 | 2.3E-190 |
| ANP32E    | 81611  | 3446   | 1085.54       | 125.58         | −2.450521296        | 1.8879E-119 | 6.6251E-121 | 207.8         | −2.73837807         | 3.3E-188 | 1.8E-189 |
| GBA       | 2629   | 2637   | 795.64        | 60             | −3.067868433        | 7.1629E-111 | 2.7981E-112 | 422.83        | −1.26527081         | 3.31E-50 | 8.35E-51 |
| HOXC5     | 3222   | 1645   | 592           | 1              | −8.548240396        | 5.0096E-96 | 2.4502E-97 | 18            | −5.397261106        | 7.7E-159 | 4.9E-160 |
| HOXC4     | 3221   | 1689   | 1196          | 281            | −1.428362385        | 2.4963E-64 | 2.1899E-65 | 323           | −2.241844062        | 3E-165  | 1.9E-166 |
| PRR5-ARHGAP8 | 553158 | 1179   | 342.35        | 11.31          | −4.258588209        | 1.9348E-61 | 1.8126E-62 | 97.19         | −2.169824969        | 1.43E-46 | 4.03E-47 |
| POR       | 5447   | 2509   | 860.3         | 170.31         | −1.675463721        | 1.4554E-58 | 1.4358E-59 | 216.96        | −2.340643496        | 1.6E-125 | 1.3E-126 |
| MTPN      | 136319 | 3900   | 314.21        | 0              | −8.634372317        | 3.9070E-51 | 4.8762E-52 | 0             | −9.648818028        | 1.19E-62 | 2.22E-63 |
| PMF1      | 11243  | 1122   | 1023.19       | 273.66         | −1.241404619        | 7.8482E-45 | 1.2185E-45 | 641.82        | −1.02606616         | 2.8E-46  | 7.9E-47  |
| CDRT4     | 284040 | 2515   | 243.61        | 0              | −8.267216577        | 1.3382E-41 | 2.3928E-42 | 0             | −9.281662288        | 2.29E-51 | 5.62E-52 |
| SDSL      | 11365  | 1311   | 325           | 36             | −2.513157937        | 7.6649E-38 | 1.6162E-38 | 40            | −3.357600555        | 1.42E-69 | 2.35E-70 |
| CDKN2A    | 1029   | 1218   | 1132          | 351            | −1.028118053        | 6.0706E-37 | 1.3083E-37 | 191           | −2.920462157        | 2E-209  | 9.4E-211 |
| PCSK1N    | 27344  | 1071   | 855           | 238            | −1.183748977        | 3.8728E-35 | 9.0900E-36 | 178           | −2.617279921        | 1.5E-141 | 1.1E-142 |
| SURF1     | 6834   | 1046   | 189.76        | 0              | −7.906819136        | 6.4156E-34 | 1.6099E-34 | 34            | −8.921264847        | 3.2E-42  | 1.05E-42 |
| ZNF616    | 90317  | 4386   | 295.55        | 35.94          | −2.378526937        | 1.4846E-32 | 4.0016E-33 | 18            | −4.390566151        | 3.72E-75 | 5.49E-76 |

*aGene length; bgene expression of group NC; clog2 transformed fold change between NC group and sh1 group; dadjusted p value; ep value
Fig. 2  GO classification of DEGs. a NC vs sh1; b NC vs sh2. GO classification and functional enrichment among molecular biological functions, cellular components and biological processes. X axis represents the number of DEGs. Y axis represents GO terms. c NC vs sh1; d NC vs sh2. GO classification of upregulated and downregulated genes. X axis represents GO terms. Y axis represents the amount of up- or down-regulated genes.
Fig. 2 (continued)
Fig. 2 (continued)
inhibit mTORC1 signaling, and the combined inhibition of mTORC1 and MNK could totally abrogate the growth of CTCL cells [18]. Taking together, these findings could help to understand the mechanism of action of TOX in CTCL and provide clues to novel therapeutics for CTCL.

Several strategies have been employed to enhance the efficacy of current treatments and to find new therapeutic options to improve survival and quality of life for patients with SS and other forms of advanced CTCL [19, 20, 30]. TOX encodes a high-mobility group family (HMG) domain binding nuclear protein which regulates the differentiation of developing T cells. It is thought of as a molecular marker for histological diagnosis of CTCL [6, 31]. Our work has addressed the role of DEGs after TOX knockdown, as GO functional enrichment and pathway analysis have indicated. A limitation of this work is that findings so far are restricted to a single cell line. However, we believe the results may provide some insights into the mechanism of TOX in CTCL as well as candidate targets for therapy of CTCL in the near future.

Fig. 3 Pathway functional enrichment of DEGs between group NC and group sh1. a Pathway classification of DEGs; b Pathway functional enrichment of DEGs. c Pathway functional enrichment results for up- or down-regulated genes. X axis represents the term of pathways. Y axis represents the number of up- or down-regulated genes.
Fig. 3 (continued)
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Compliance with ethical standards

Conflict of interest  The authors state no conflict of interest.

Ethical approval  This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 3  Top ten KEGG pathways with a high representation of DEGs

| Pathway                  | DEGs genes with pathway annotation (5617) | All genes with pathway annotation (23,480) | p value       | q value       | Pathway ID |
|--------------------------|-------------------------------------------|-------------------------------------------|---------------|---------------|------------|
| Pathways in cancer       | 1235 (19.8%)                              | 3097 (13.19%)                             | 8.962456e-68  | 4.959226e-66  | ko05200    |
| Melanogenesis            | 847 (13.58%)                              | 1946 (8.29%)                              | 3.684796e-64  | 1.595685e-62  | ko04916    |
| Wnt signaling pathway    | 856 (13.72%)                              | 2018 (8.59%)                              | 8.728826e-59  | 2.069979e-57  | ko04310    |
| Breast cancer            | 901 (14.44%)                              | 2066 (8.8%)                               | 4.650451e-69  | 3.087899e-67  | ko05224    |
| Gastric cancer           | 918 (14.72%)                              | 2205 (9.39%)                              | 8.712956e-59  | 2.069979e-57  | ko05226    |
| Proteoglycans in cancer  | 974 (15.61%)                              | 2264 (9.64%)                              | 2.275643e-71  | 1.888784e-69  | ko05205    |
| Hepatocellular carcinoma | 890 (14.27%)                              | 2094 (8.92%)                              | 9.088525e-56  | 2.743082e-60  | ko05225    |
| Hippo signaling pathway  | 879 (14.09%)                              | 2041 (8.69%)                              | 3.845024e-64  | 1.595685e-62  | ko04390    |
| Signaling pathways regulating pluripotency of stem cells | 860 (13.79%) | 1999 (8.51%) | 2.096168e-56  | 6.959278e-61  | ko04550    |
| mTOR signaling pathway   | 861 (13.8%)                               | 2026 (8.63%)                              | 1.376305e-59  | 3.807777e-58  | ko04150    |
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