Pathology - Research and Practice 215 (2019) 152494

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Integrating microRNA and mRNA expression in rapamycin-treated T-cell acute lymphoblastic leukemia

Xi Chen[a,b], Zhibo Guo[a], Shengjin Fan[a], Lili Sun[a], Huibo Li[a], Jin Zhou[a], Yinghua Li[a,*]

[a] Department of Hematology, The First Affiliated Hospital, Harbin Medical University, Harbin, China
[b] Department of Hematology, The Second Affiliated Hospital, Harbin Medical University, Harbin, China

ARTICLE INFO

Keywords:
T-cell acute lymphoblastic leukemia
Rapamycin
Cell survival pathways
Regulatory network
miRNA

ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) has a relatively improved remission rate, but the poor outcomes are primarily due to resistance and relapse. Moreover, organs infiltration trends to occur during remission. Rapamycin was applied to treat malignancies for decades. In this investigation, we aimed to explore the molecular mechanisms and pathway changes during the T-ALL therapeutic process. T-ALL cell line Molt-4 cells were treated with rapamycin and performed microarray analysis to identify the deregulated miRNAs and mRNAs (log2 fold change > 2 or < -2). To obtain regulatory miRNA/mRNA network, miRNA target prediction softwares and Cytoscape were used to plot and modularize the rapamycin treatment-related network. Surprisingly, the enriched pathways were not involved in mediating either cell death or apoptosis but were responsible for angiogenesis, cell survival, and anti-apoptosis, which is consistent with the Gene Ontology analysis and PPI network based on all deregulated mRNAs, indicating that these elements likely play a role in promoting Molt-4 cell survival or escaping from rapamycin. The expression of 3 miRNAs (miR-149-3p, miR-361-3p, and miR-944) and their putative targets, which play central roles in their module, were validated by qRT-PCR. These results provide novel insight into potentially relevant biological pathways for T-ALL cells escaping from chemotherapy or developing central nervous system infiltration.

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy that accounts for approximately 15% of pediatric and 25% of adult acute lymphoblastic leukemia (ALL) cases and is generally associated with unfavorable clinical features and invasive biological behavior, such as resistance, early relapse and isolated central nervous system relapse [40]. Although T-ALL is more commonly diagnosed in children, who accounted for nearly 60% of new cases in 2015, the outcomes in adults are much worse as follows: nearly 80% of ALL-related deaths occurred in adults. Furthermore, the prognosis is not sufficiently good considering the poor outcomes, including resistance and relapse, that are faced by both pediatric and adult patients even with intensive chemotherapy [5]. Organ infiltrations, especially central nervous system infiltration, during remission phases resulted the survival rates in some cases driven down to as low as 40% [31,41]. A better understanding of the disease is urgently desired; in particular, studies performing molecular analyses, such as investigations based on the altered pathways in T-ALL after treatment with chemotherapy, are needed. Evidence suggests that numerous pathways are involved in the pathogenesis, resistance and relapse of T-ALL, and the NOTCH1, PI3K/Akt/mTOR, and NF-κB pathways have attracted considerable interest [6,24].

MicroRNAs (miRNAs), which are characterized as small (19–25 nt), non-coding RNAs, function as negative posttranscriptional regulators of messenger RNAs (mRNAs) by binding to the 3′ untranslated region (UTR) [3]. miRNAs play vital roles in physiological and pathological processes. Alterations in miRNAs may have a great impact on the pathogenesis of multiple diseases as both tumor suppressors and oncogenes [2,16]. Previous studies have implicated aberrant miRNA expression in multiple hematological malignancies [10,17]. Interestingly, miRNA expression has been reported to be associated with drug resistance in pediatric ALL [45], and dysregulated miRNA networks have already been associated with T-ALL [32,44].

Similar to multiple proteins acting in concert via various pathways, numerous miRNAs may cooperatively modulate the same target genes, and individual miRNAs have the potential to target hundreds of genes [48]. Due to this diversity, thoroughly characterizing miRNA-mRNA

* Corresponding author at: Department of Hematology, The First Affiliated Hospital, Harbin Medical University, 23 Youzheng Street, Nangang District, Harbin, 150001, China.
E-mail address: yinghualihmu@126.com (Y. Li).

https://doi.org/10.1016/j.prp.2019.152494
Received 14 April 2019; Received in revised form 24 May 2019; Accepted 8 June 2019

regulatory networks is challenging. We can utilize emerging bioinformatics tools and algorithms, such as TargetScan and PicTar [19,34], to obtain insight into the vast landscape of miRNA-mRNA relationships.

Rapamycin, which is an mTOR pathway inhibitor, has been applied as an antineoplastic agent alone or in combination with other classical anti-tumor drugs for decades. Our previous study demonstrated that in ALL cell lines (Molt-4 and Nalm-6), mTOR was highly expressed. Rapamycin restored the expression of p14, p15 and p57 in these cells to arrest cell cycle at G1/S phase [25]. Rapamycin was also used combined with glucocorticoids, imatinib, and doxorubicin to reverse multidrug resistance in ALL [25]. Due to the high frequency of PI3K/Akt/mTOR pathway activation in T-ALL, clinical trials of rapamycin and derivative in ALL were conducted in multiple clinical centers [11,38,42]. However, the results were not desirable [38,42]. The mechanisms underlying the reduced sensitivity and resistance include alterations, such as somatic TSC1 mutations and mTOR mutations, and aberrant activated pathways substituting for the effect of mTOR [23,52]. Therefore, studies investigating rapamycin-related pathway alterations, such as the aberrantly activated AKT signaling pathway, are desperately needed [56].

In the current investigation, we aimed to identify the molecular mechanisms underlying T-ALL treatment by rapamycin via analyzing altered pathways mediated by differentially expressed miRNAs and mRNAs. miRNA and mRNA microarrays were performed using the T-ALL cell line Molt-4 after rapamycin or control treatment. A miRNA-mRNA regulatory network was constructed based on the microarray data. This study depicts the roles of the miRNA-mRNA regulatory network in the pathways altered by the treatment with rapamycin, and the results provide novel insight into potentially relevant biological pathways for T-ALL cells escaping from chemotherapy or developing central nervous system infiltration.

2. Materials and methods

2.1. Cells, cell culture and reagents

The T-ALL cell lines Molt-4, Jurkat, CCRF-CEM and KOPTK1 were purchased from ATCC (Manassas, VA, USA) and maintained in RPMI1640 medium (HyClone, UT, USA) with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, UT, USA). Rapamycin (Sigma, MO, USA) was dissolved in 100% DMSO to a stock concentration of 10^{-2} M and stored at −20 °C. As our previous study, Molt-4 cells in the treated group were incubated with rapamycin (100 nM) for 24 h at 5% CO_2 and 37 °C, while the control group was maintained under the same conditions but without rapamycin [25].

2.2. Total RNA extraction

The total RNA was isolated from the samples of both groups using TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions. The RNA quality and quantity were accurately measured using a NanoDrop spectrophotometer (ND-1000, Nanodrop Technologies, DE, USA). The RNA integrity was assessed by gel electrophoresis (Fig. S1).

2.3. Microarray experiments and data analysis

The details of the microarray experiments and data analysis are described in the Supplementary materials.

2.4. Identification of dysregulated miRNA-mRNA regulatory pairs

The potential target genes of all dysregulated miRNAs were identified using seven miRNA prediction databases (TargetScan, PicTar, DIANA-microT [37], miRTarBase [9], RNA22 [28], MIRDB [54], and miRanda [4]). Only genes uniformly predicted by 3 of these seven alghorithms were considered target genes of the given miRNAs and included in the following analysis [12,57].

Overlapping sets of predicted genes and dysregulated mRNAs were obtained. The pairs of inversely expressed miRNA and mRNAs were then identified; the up-regulated miRNAs were paired with the down-regulated mRNAs, and the down-regulated miRNA were paired with the up-regulated mRNAs.

2.5. Construction and modularization of the rapamycin treatment-related gene regulatory network

Cytoscape software (version 3.6.0; www.cytoscape.org) was used to construct the rapamycin treatment-related gene regulatory network, which contained the identified inversely correlated pairs [49].

To visualize and query the cooperativity in biological processes among the miRNA classes in the integrated miRNA-mRNA matrix, miRNA functional modules were constructed [7]. The modularization was performed using the auxiliary function EAGLE algorithm in Cytoscape (Options: CliqueSize Threshold: 3, ComplexSize Threshold: 2).

2.6. Functional annotation

To confirm the pathway alterations after the rapamycin treatment, we conducted a Gene Ontology (GO) analysis based on all differentially expressed mRNAs using the Database for Annotation Visualization and Integrated Discovery (DAVID version 6.8; https://david.ncifcrf.gov/) to corroborate the representativeness of the miRNA function modules in biological processes [22]. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was also applied to all differentially expressed genes to further obtain the protein-protein interaction network (PPI network) [51].

A KEGG pathway analysis was performed using the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database to analyze the gene sets in each module rather than all genes in the entire network.

2.7. RNA extraction and quantitative real-time PCR (qRT-PCR)

The expression of 3 miRNAs (miR-944 in module 1, miR-149-3p and miR-361-3p in module 2) and 5 mRNAs (*THBS1* in module 1 and *LTBR*, *EGFR* and *VEGFC* in module 2) were measured by qRT-PCR. The total RNA from 4 T-ALL cell lines (Molt-4, Jurkat, CCRF-CEM and KOPTK1) was extracted after treatment with rapamycin or control cell culture. cDNA was obtained using an M-MLV Reverse Transcriptase reagent kit (PROMEGA, WI, USA) according to the standard protocols. qRT-PCR was performed using an ABI PRISM 7900 sequence detection system (Barcelona, Spain), and the relative expression of the amplified RNA samples was calculated using the $2^{-\Delta\Delta}$CT algorithm. The results are presented as the fold changes in the mRNAs/miRNAs in the cell lines after the rapamycin treatment relative to the untreated samples. The primer sequences are shown in the Supplementary materials (Table S1).

2.8. Statistical analysis

The statistically significant differences between two groups were estimated by performing Student's *t*-tests using GraphPad Prism 5.0 (GraphPad Software, CA, USA). The significantly enriched GO terms and pathways were identified by performing Fisher's exact tests. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Dysregulated miRNAs and mRNAs between the treated and untreated Molt-4 cells

We compared the miRNA and mRNA profiles in the treated and untreated groups according to microarray results, and all deregulated

miRNAs and mRNAs were selected by the log2 fold change (> 2 or < -2), $p < 0.05$.

In total, 71 dysregulated miRNAs were observed in the treated group compared to those in the untreated group; 32 miRNAs were up-regulated, and 39 miRNAs were down-regulated (Table S3). We detected 136 differentially expressed mRNAs between the treated and untreated groups, including 109 up-regulated mRNAs and 27 down-regulated mRNAs in the treated group (Table S4).

3.2. Identification of regulatory pairs of deregulated miRNAs and target mRNAs

The putative target genes of 71 dysregulated miRNAs were obtained, and these findings were integrated with the above data of the differentially expressed mRNAs (Fig. 1). Since one miRNA may target multiple mRNAs, and single mRNA can also be targeted by more than one miRNAs, we obtained 200 pairs involving 37 miRNAs and 65 mRNAs with inverse expression relationships.

3.3. Construction and modularization of a rapamycin treatment-related gene regulatory network

A rapamycin treatment-related gene regulatory network was constructed based on the identified miRNA-mRNA pairs to investigate the regulatory effects of these negative relationships (Fig. 1A). A KEGG pathway enrichment analysis of the entire network was conducted.

Then, the network was artificially subdivided into 6 polycentric modules as stated [7], containing multiple miRNAs as centers regulating 6–18 genes, and these modules had strong interactions with each other (Fig. 1B, Table S5).

3.4. Functional enrichment analysis

To investigate the functions of the deregulated mRNAs and miRNA-mRNA pairs in the network, a GO analysis and PPI network were conducted on all deregulated genes, and a KEGG pathway enrichment analysis was performed based on the gene sets in each module.

Surprisingly, the enriched pathways were not involved in mediating cell death, apoptosis, or other cell death pathways, but these pathways modulated angiogenesis (HIF-1sinaling pathway, PI3K/Akt signaling pathway, Focal adhesion and Pathways in cancer; Figs. S5–S8), cell proliferation and survival (such as Ras and PI3K/Akt signaling pathway; Figs. S2–S8), and motility (Focal adhesion and Pathways in cancer; Figs. S2&S8).

The genes included in the GO analysis are enriched in Focal adhesion, the PI3K-Akt signaling pathway, proteoglycans in cancer, etc. (Fig. 2A).

The PPI networks are enriched in pathways related to angiogenesis and cell migration, such as blood vessel development, extracellular matrix organization, etc. (Fig. 2B, the entire PPI pathway is shown in Table S6)

Then, a KEGG pathway enrichment analysis was performed on all genes in each module. In total, 4 and 10 pathways were enriched in module 1 and 2, respectively, while the gene sets in the other 4 modules did not show enrichment in any of the distinct KEGG pathways (Table 1). The enriched KEGG pathways in module 1 contain focal adhesion, proteoglycans in cancer, and microRNAs in cancer, and the enriched pathways in module 2 include the PI3K-Akt signaling pathway, focal adhesion, and pathways in cancer, which is consistent with the results of the GO analysis.

Consequently, we focused on these 2 modules and mapped the relevant proteins corresponding to the network to these enriched pathways.

3.5. Annotation of enriched KEGG pathways and proteins related to cell survival in module 1

In module 1, 4 enriched KEGG pathways were identified; 2 pathways were of interest, and the other 2 pathways were specific to cancer but not leukemia (Table 1). The 2 meaningful pathways, i.e., focal adhesion and proteoglycans in cancer, were further analyzed (Figs. 3A & S2). The key miRNA in module 1 was miR-944, which targets *THBS1* synergistically with miR-29b-1-5p. Thrombospondin-1, which is the protein encoded by the *THBS1* gene, and caveolin-1, which is encoded by *CAV1*, are involved in the focal adhesion pathway modulating angiogenesis and T-ALL cell mobility, which might promote infiltration. In the proteoglycans in cancer pathway, 3 proteins, i.e., homeobox D10 (HOXD10), thrombospondin-1 and caveolin-2, negatively contributed to cell growth, migration and invasion, suggesting that the rapamycin-treated Molt-4 cells had poor proliferation. Matrix metalloproteinase-2 (MMP2) had an effect opposite to THBS1, inhibiting the survival of T-ALL cells as MTT shown [25].

The two enriched pathways in module 1 appear to help leukemia cells survive at the cost of proliferation and motility, indicating that cells evade rapamycin by activating these two identified pathways. However, the outcomes are generated by proteins with divergent and, in certain cases, antagonistic functions. This issue along with the findings in module 2 are further elaborated upon in the discussion.

3.6. Annotation of enriched KEGG pathways and proteins related to cell survival in module 2

In module 2, 8 annotated pathways were closely related to lymphocyte adhesion, cell survival, and angiogenesis (i.e., Ras signaling pathway, Rap1 signaling pathway, cytokine-cytokine receptor interaction, HIF-1 signaling pathway, PI3K/Akt signaling pathway, focal adhesion, pathways in cancer, and NF-kappa B signaling pathway). The five-star genes involved in these 8 enriched KEGG pathways in module 2 encode epidermal growth factor receptor (*EGFR*), vascular endothelial growth factor C (*VEGFC*), lymphotoxin beta receptor (*LTBR*), metallopeptidase inhibitor 1 (*TIMP1*), and intercellular cell adhesion molecule-1 (*ICAM1*) (Figs. 3B and S3–9). EGFR is localized on the cell surface and, thus, plays a pivotal role in angiogenesis and the proliferation, differentiation, and survival of multiple cell types. When cells are stressed, such as following chemotherapy treatment, EGFR induces cell migration [58] (Figs. 3B and S3–8). VEGFC functions specifically as a growth factor in endothelial cells and contributes to angiogenesis, cell growth and cell proliferation [47] (Figs. 3B and S2–3, 5–8). LTBR, which is involved in the HIF-1 and NF-kappa B signaling pathways, participates in the development of lymphoid tissue and transformed cells and helps trigger apoptosis, which antagonizes the actions of the above two proteins; these functions are similar to those of MMP2 in module 1 (Figs. 3B and S5 & 8). The negative regulators of *LTBR* mRNA in our network were miR-4299 and miR-149-3p, which were associated with a poor prognosis in T-ALL [28]. TIMP1, which inhibits the degradation of the extracellular matrix by matrix metallopeptidase, also has anti-apoptotic activity (Fig. S5). Previous studies have reported the role of TIMP1 in lymphoma cell chemoresistance under conditions of DNA damage [21]. Finally, ICAM1, which is also known as CD54, has the ability to mediate intercellular conglutination and promote leukocyte migration [15] (Fig. S8). The up-regulation of *ICAM1* mRNA expression was probably related to central nervous system leukemia after chemotherapy. These strategies appear to suppress miR-149-3p and miR-4299, thus favoring pathways that promote apoptosis.

3.7. Validation of key miRNAs and mRNAs by qRT-PCR in T-ALL cell lines

The expression of miRNAs with over 12 targets along with mRNAs predicted to be targeted in module 1 and module 2 were validated via qRT-PCR in 4 T-ALL cell lines. We observed a similar up- and down-

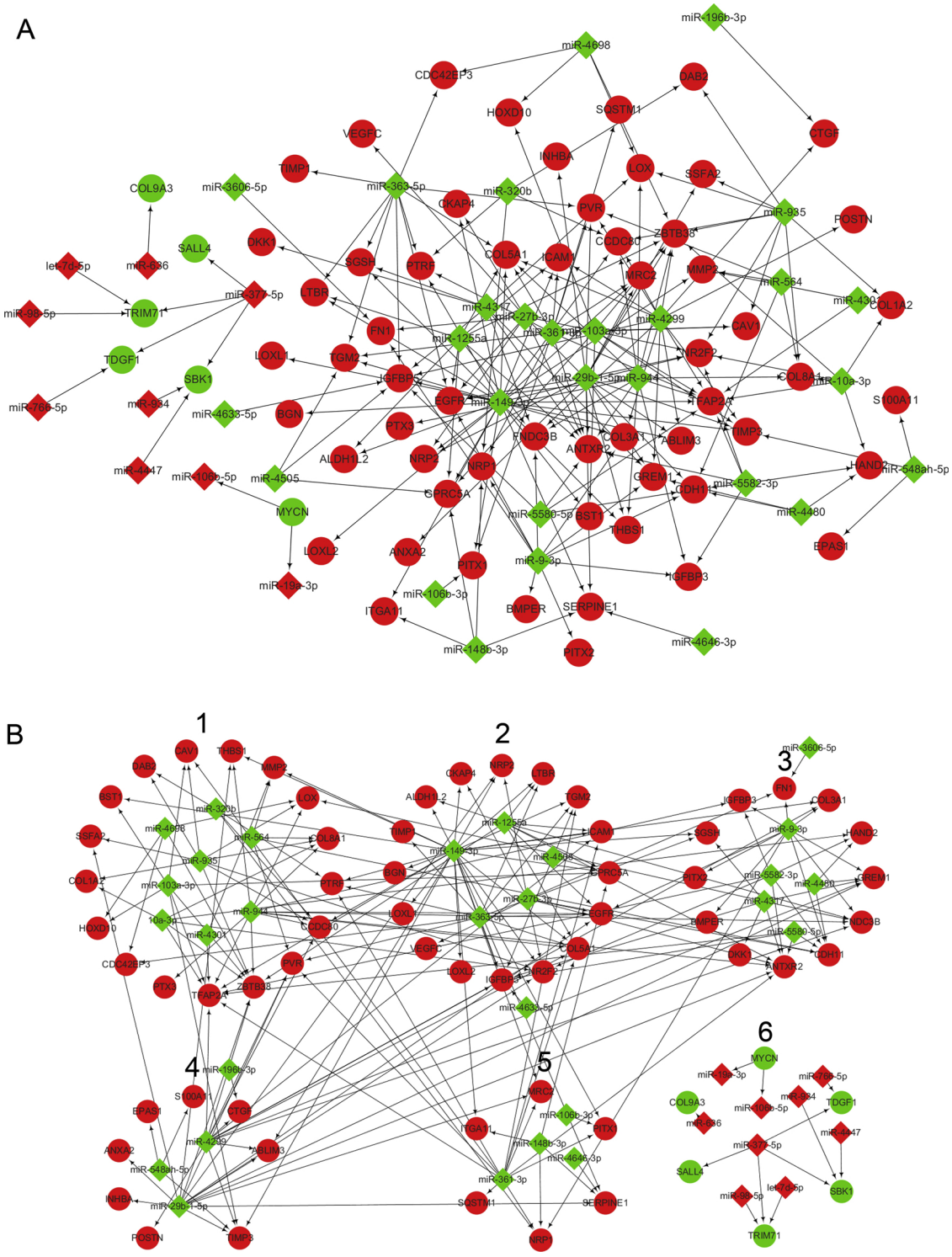

Fig. 1. Construction and modularization of the rapamycin treatment-related gene regulatory network.
A. The whole network of rapamycin treatment-related miRNA-mRNA regulatory pairs. 200 regulatory pairs involving 37 miRNAs and 65 mRNAs were identified. In this figure, diamond nodes represent the differentially expressed miRNAs, and round nodes represent differentially expressed mRNAs targeted by the miRNAs. Green and red nodes indicate that the miRNAs or mRNAs were down-regulated and up-regulated, respectively. The arrows indicate that the miRNAs can regulate the expression of the target mRNAs. B. The network was subdivided into 6 modules. In this figure, regulatory pairs of miRNA and mRNA were divided into 6 modules according to the links between nodes. The numbers are serial numbers of each module.

regulation profile in the Molt-4 cells in the microarray and qRT-PCR data. In the other 3 cell lines, miR-149-3p (in KOPTK1 and CCRF-CEM), *THBS1* (in KOPTK1), *CAV1* (in KOPTK1 and CCRF-CEM), and *VEGFC* (in Jurkat, KOPTK1 and CCRF-CEM) presented a different regulation profile tendency (Fig. 4).

4. Discussion

As previously mentioned, resistance and relapse greatly contribute to the poor prognosis of T-ALL, CNS infiltration especially. Although intensive chemotherapy, allogeneic hematopoietic stem cell

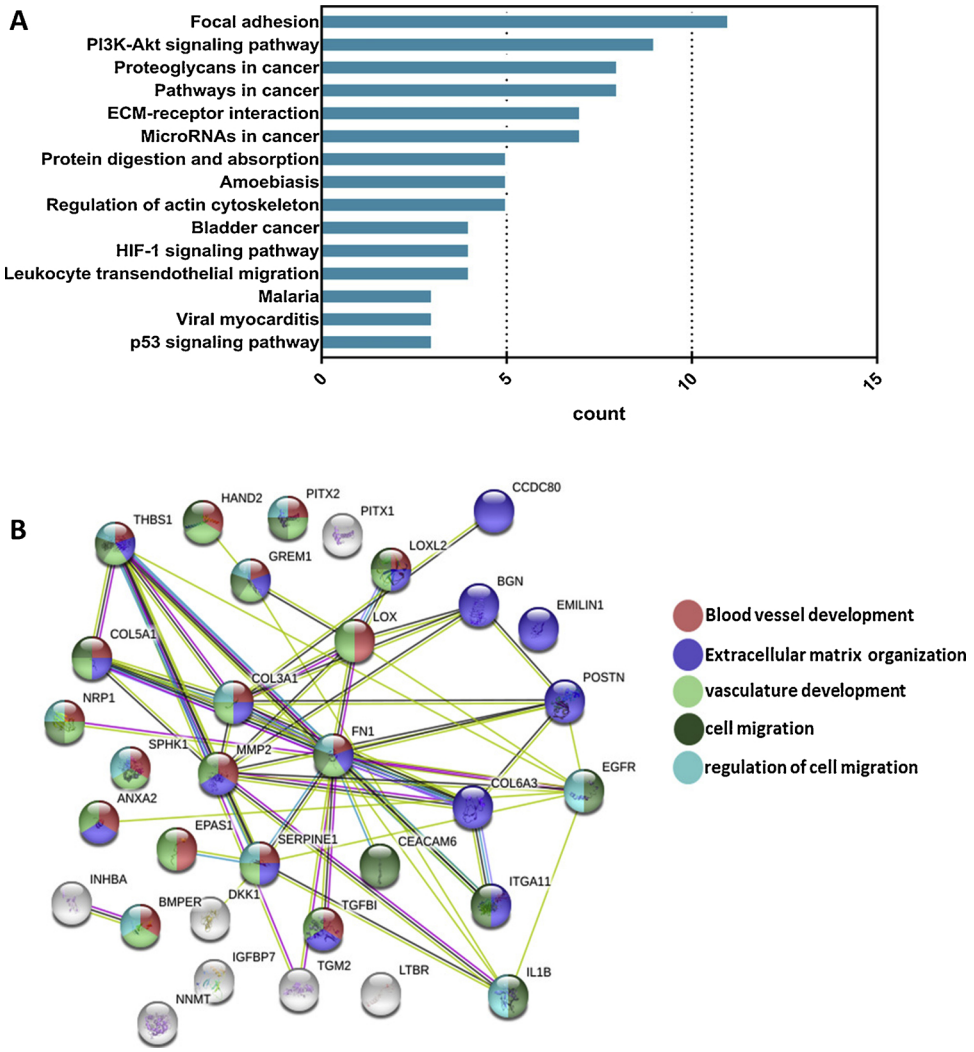

Fig. 2. GO analysis and PPI network of all differentially expressed mRNAs.

A. GO analysis of all differentially expressed mRNAs was conducted. The top 10 enriched pathways are listed in gene counts order, i.e., focal adhesion, PI3K-Akt signaling pathway, proteoglycans in cancer, pathways in cancer, ECM-receptor interaction, microRNAs in cancer, protein digestion and absorption, amoebiasis, regulation of actin cytoskeleton, and bladder cancer. Pathways related to leukemia and cell survival were annotated further.

B. PPI network of all proteins encoded by all differentially expressed mRNAs was constructed. Each node represents a protein. Red nodes represent proteins enriched in blood vessel development, FDR = 1.09E-16; mazarine nodes represent proteins enriched in extracellular matrix organization, FDR = 1.09E-16; aqua nodes represent proteins enriched in vasculature development, FDR = 1.37E-16; azure and bottle green nodes represent proteins enriched in cell migration, FDR = 3.77E-07, and regulation of cell migration, FDR = 5.43E-07, respectively.

transplantation (allo-HSCT), and even clinical trials are improving daily, the outcomes of refractory or relapsed T-ALL patients remain dismal [59]. Previous studies on CNS infiltration settled down in cytokines including CXCL12/CXCR4-signaling [31], cell signaling molecules such as PKCθ [35].

In our current study, the miRNA and mRNA expression profiles were integrated and analyzed to determine the behavior of the T-ALL cell line Molt-4 after a treatment with rapamycin to explore the mechanisms of drug activity or failure. Module 1 enriched PI3K/Akt pathways suggested downstream mTOR pathway was indeed blocked. However, activated survival pathways failed to work *ex vivo*, and we will discuss later. Moreover, a rapamycin treatment-related network was created with only miRNAs and mRNAs. Transcription factors (TFs) are crucially connected to the regulation of miRNAs, which modulate mRNAs at the post-transcriptional level [12,57]. We only identified one TF (calmodulin binding transcription activator 1, CAMTA1, 2.29 fold down-regulated after rapamycin treatment, data not shown) that was relevant to the dysregulated miRNAs (miR-9-3p [46]), and this TF was not

Table 1
Functional analysis of the modules using the KEGG database.

No.	Module	Enriched KEGG pathway	Genes
1	1	Focal adhesion (Supplementary Fig. 2)	THBS1, CAV1
2	1	Proteoglycans in cancer (Fig. 3A)	THBS1, MMP2, HOXD10, CAV1
3	1	MicroRNAs in cancer	THBS1, HOXD10
4	1	Bladder cancer	THBS1, MMP2
5	2	Ras signaling pathway (Supplementary Fig. 3)	EGFR, VEGFC
6	2	Rap1 signaling pathway (Supplementary Fig. 4)	EGFR, VEGFC
7	2	Cytokine-cytokine receptor interaction (Fig. 3B)	EGFR, LTBR, VEGFC
8	2	HIF-1 signaling pathway (Supplementary Fig. 5)	EGFR, LTBR, TIMP1
9	2	PI3K-Akt signaling pathway (Supplementary Fig. 6)	EGFR, VEGFC
10	2	Focal adhesion (Supplementary Fig. 7)	EGFR, VEGFC
11	2	Pathways in cancer (Supplementary Fig. 8)	EGFR, VEGFC
12	2	NF-kappa B signaling pathway (Supplementary Fig. 9)	ICAM1, LTBR
13	2	AGE-RAGE signaling pathway in diabetic complications	ICAM1, VEGFC
14	2	HTLV-I infection (Supplementary Fig. 10)	ICAM1, LTBR

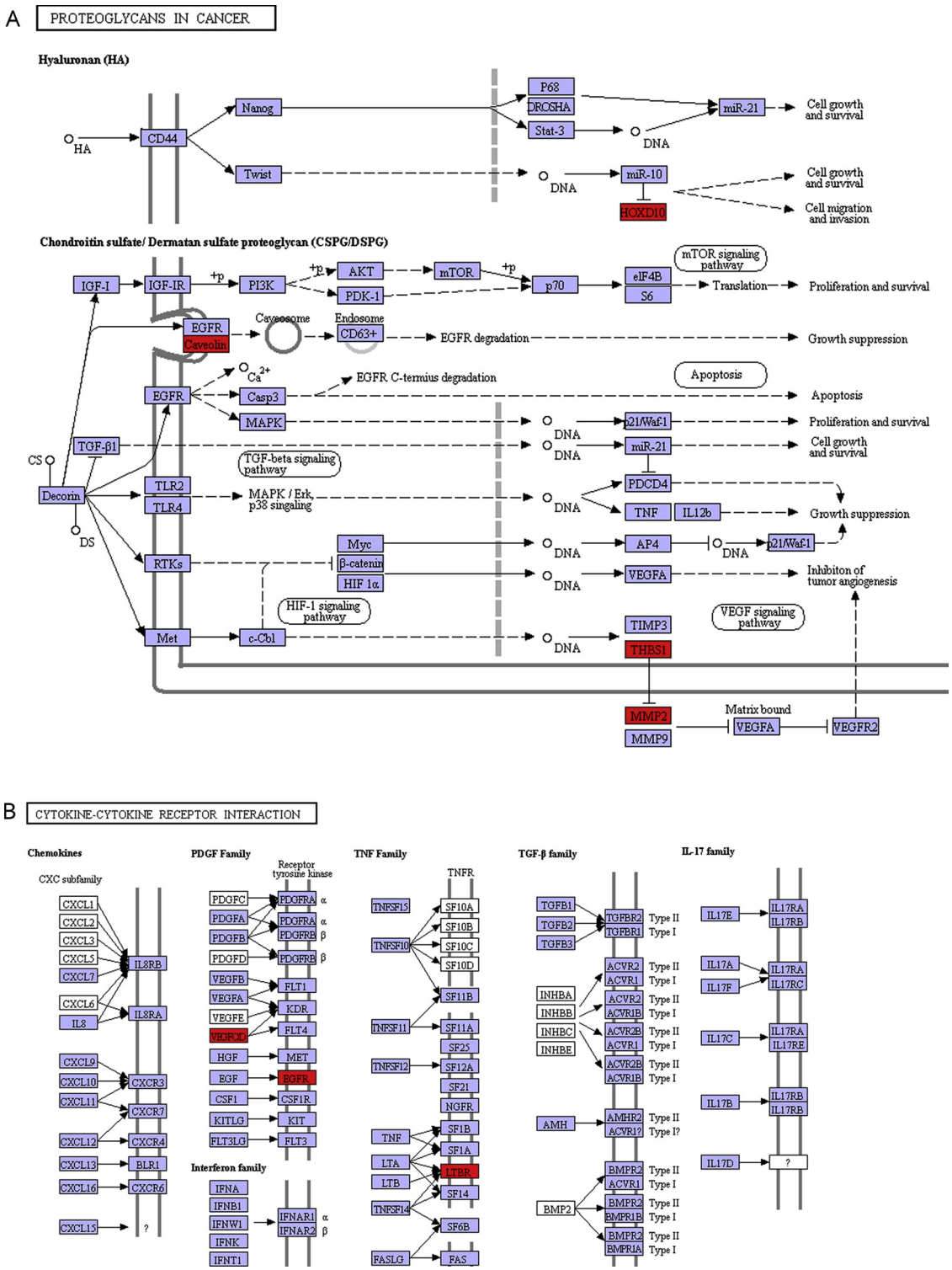

Fig. 3. Maps indicating enriched KEGG pathways of genes in module 1.
A. Proteoglycans in cancer pathway in module 1.
B. Cytokine-cytokine receptor interaction in module 2.
Each box in the map indicates the placement of a gene in the pathway. The red boxes indicate the up-regulated genes in the treated group compared to the untreated group.

structured in the network. The generated network will become more complete upon the addition of differentially expressed TFs and cytokines.

The 6 modules in the rapamycin treatment-related network were structurally connected to each other, and miRNAs and mRNAs in the same module and separate modules had relatively strong relationships. Thus, to a certain extent, the elements within the same module often exhibit much tighter relationships that are both structural and functional [33]. Therefore, we could consider the biological network module a comparatively independent functional unit.

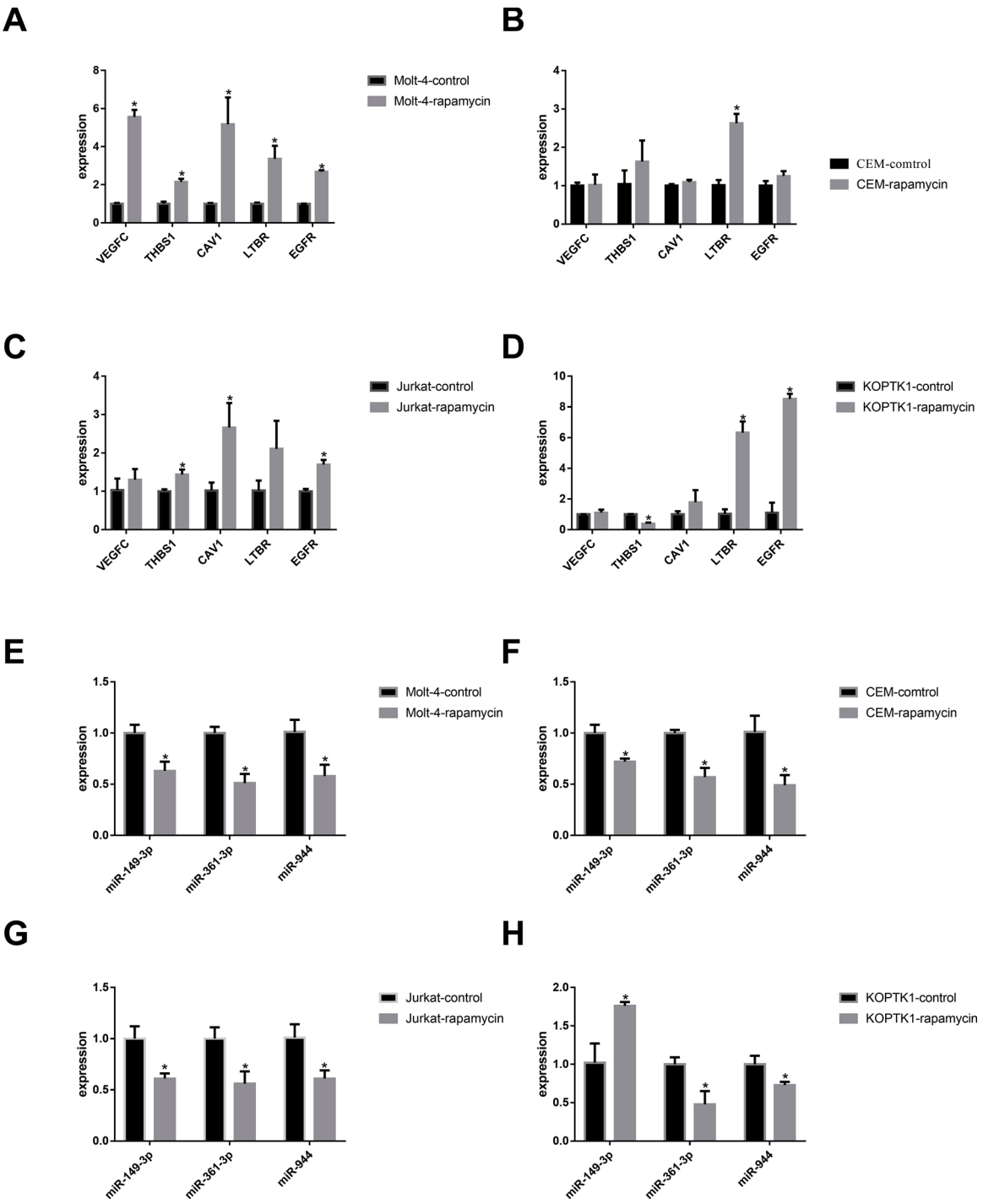

Fig. 4. RNA expression validated by qRT-PCR in 4 T-ALL cell lines.
The expression of 5 mRNAs (*VEGFC*, *THBS1*, *CAV1*, *LTBR* and *EGFR*, A–D) and 3 miRNAs (miR-149-3p,miR-361-3p and miR944, E–H) in Molt-4 (A&E), CCRF-CEM (B &F), Jurkat (C&G) and KOPTK1 (D&H) cells after a treatment with rapamycin compared with that in the untreated cells. The data are presented as the means ± SD of three separate experiments. * indicates $P < 0.05$.

miR-149-3p, miR-361-3p, and miR-944 played critical roles in modules they belonged to. With bioinformatic analysis in our work and other investigations previously, evidences were clear on the drug resistance connection for these three miRNAs. In module 1, down-regulated miR-944 was expected to play critical functions in resistance for its putative target genes activating survival pathways as mentioned in the part of Result earlier. miR-944 also works as tumor-suppressors in hepatocellular carcinoma and gastric cancer by deactivating the PI3K/Akt or MACC1/Met/Akt signaling pathway [29,36], in lung adenocarcinoma by targeting STAT1 interaction [1]. Furthermore, the decreased expression of miR-944 leading to cell migration in breast cancer cells [18] and metastasis in colorectal cancer patients [53]. miR-149-3p (once known as miR-149*) was the critical functional core of module 2, and the aggressive outcomes due to its predicted targets (e.g. ICAM-1 and LTBR) were responsible for rapamycin resistance. The expression of miR-149-3p inhibits proliferation, migration and invasion of bladder and breast cancer cells [13,55], also exerts as inhibitor of Akt1 signaling pathway in multiple human cancer cells [26,50]. Though

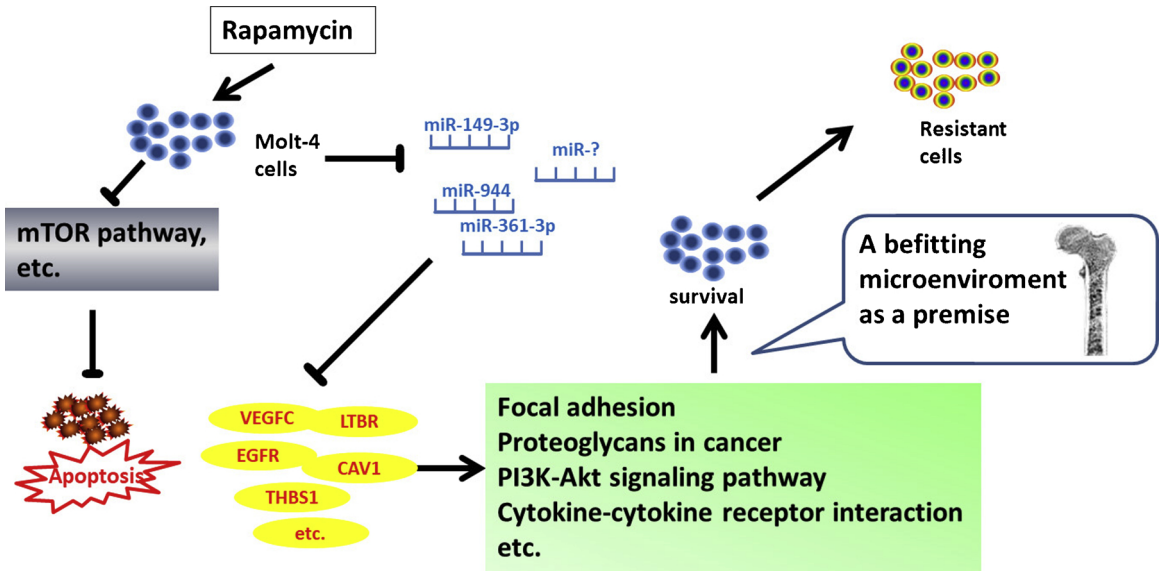

Fig. 5. Putative drug resistance pathways require drug stress as a 'switch'.
A chemotherapy insult to Molt-4 cells acts as a 'switch' that activates certain pathways and promotes leukemia cells to release cytokines or miRNAs that signal to the surrounding microenvironment for help. A lack of a befitting microenvironment as *in vivo* leads to a failure in activating the requisite pathways, even though the key molecules in the pathways are already available.

miR-361-3p was drawn into module 5, there were overlapping putative target mRNAs between miR-149-3p and miR-944, such as *ICAM1*, *PTRF* and *TGM2* (Fig. 1B). miR-361-3p was identified as a tumor-suppressor in non-small cell lung cancer bound by circular RNA 100146 [8], and the high expression of miR-361-3p was associated with better outcome of cervical cancer [27] and non-small cell lung cancer patients [43]. We hypothesize that rapamycin stress acts as a switch to decrease the expression of miR-944, miR-149-3p and miR-361-3p, which in turn abolished the inhibition of these 3 miRNAs on survival, motility pathways including Akt signaling and other "protective" pathways that activate cell survival and migration (Fig. 5).

As previously mentioned, the annotation of the proteins in the two active modules yielded interesting disagreements. Chemotherapy is always more effective at killing *in vitro* than *in vivo*. In addition, although the annotation suggested that the Molt-4 cells were likely to benefit in terms of survival from the effects of many of the identified pathways, such as those that led to the down-regulation of miR-944 or miR-29b-1-5p, leukemia cell growth was not favorable [25]. Podshivalova also found that tumor suppressor miR-150 was restored in Jurkat cells after treated with rapamycin [39]. While, clinical trials employing rapamycin for relapsed and refractory T-ALL patients didn't work well [38,42]. One key point is that the Molt-4 culture conditions in our current study do not accurately mimic the physiological microenvironment in which leukemia cells reside with multiple types of stromal cells, including mesenchymal and blood vessel endothelial cells [14]. The leukemia cell microenvironment accounts for the resistance and anti-apoptotic activity of the rapamycin-treated Molt-4 cells as shown in the ECM-receptor interaction pathway from the GO analysis and angiogenesis shown in STRING analysis. For example, VEGFC induces the development of epithelial cells and angiogenesis, thus indirectly promoting leukemia cells [30]. In such a circumstance, miRNAs function as messengers in the crosstalk within the leukemia microenvironment that directly mediates drug resistance [20].

Further studies may focus on the requirement of these elements in the local microenvironment and combination treatments using agents that block the pathways enriched in our modules that control cell survival. The leading candidate proteins encoded by the genes in our modules are LTBR, THBS1, EGFR, VEGFC, and CAV1; however, the functions of other genes may also be important but have yet to be uncovered. Regarding the final key miRNAs in our network, it was needed to confirm their significance in drug resistance, but this confirmation will be accomplished in our future studies. Notably, we failed to validate our microarray and protein expression results in primary leukemia cells from patients; however, our data have increased our knowledge of novel mechanisms about treat failure.

5. Conclusion

Taken together, current investigation on T-ALL miRNA-mRNA alterations reveals the potential mechanisms underlying T-ALL cell survival following chemotherapy. Pathways responsible for surviving and angiogenesis were activated transcriptionally. The expression of miR-149-3p, miR-361-3p, and miR-944 together with target mRNA might play core role in chemotherapy failure or CNS infiltration.

Data availability

The datasets obtained and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Fundings

This study was funded by the Science and Technology Project of Heilongjiang Province, China, (GC12C303-4) and the Foundation for the Returned Overseas Chinese Scholars of Heilongjiang Province, China, (LCO7C22).

Acknowledgments

The authors would like to thank Dr. Liangde Xu, Department of Bioinformatics, Harbin Medical University, for the technical support.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152494.

References

[1] J.C. An, H.B. Shi, W.B. Hao, K. Zhu, B. Ma, miR-944 inhibits lung adenocarcinoma tumorigenesis by targeting STAT1 interaction, Oncol. Lett. 17 (2019) 3790–3798.
[2] S. Babashah, M. Soleimani, The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis, Eur. J. Cancer 47 (2011) 1127–1137.
[3] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[4] D. Betel, M. Wilson, A. Gabow, D.S. Marks, C. Sander, The microRNA.org resource: targets and expression, Nucleic Acids Res. 36 (2008) D149–153.
[5] R. Bortolozzi, S. Bresolin, E. Rampazzo, M. Paganin, F. Maule, E. Mariotto, D. Boso, S. Minuzzo, V. Agnusdei, G. Viola, G. Te Kronnie, G. Cazzaniga, G. Basso, L. Persano, AKR1C enzymes sustain therapy resistance in paediatric T-ALL, Br. J. Cancer 118 (2018) 985–994.
[6] D. Bressanin, C. Evangelisti, F. Ricci, G. Tabellini, F. Chiarini, P.L. Tazzari, F. Melchionda, F. Buontempo, P. Pagliaro, A. Pession, J.A. McCubrey, A.M. Martelli, Harnessing the PI3K/Akt/mTOR pathway in T-cell acute lymphoblastic leukemia: eliminating activity by targeting at different levels, Oncotarget 3 (2012) 811–823.
[7] K. Bryan, M. Terrile, I.M. Bray, R. Domingo-Fernandez, K.M. Watters, J. Koster, R. Versteeg, R.L. Stallings, Discovery and visualization of miRNA-mRNA functional modules within integrated data using bicluster analysis, Nucleic Acids Res. 42 (2014) e17.
[8] L. Chen, A. Nan, N. Zhang, Y. Jia, X. Li, Y. Ling, J. Dai, S. Zhang, Q. Yang, Y. Yi, Y. Jiang, Circular RNA 100146 functions as an oncogene through direct binding to miR-361-3p and miR-615-5p in non-small cell lung cancer, Mol. Cancer 18 (2019) 13.
[9] C.H. Chou, N.W. Chang, S. Shrestha, S.D. Hsu, Y.L. Lin, W.H. Lee, C.D. Yang, H.C. Hong, T.Y. Wei, S.J. Tu, T.R. Tsai, S.Y. Ho, T.Y. Jian, H.Y. Wu, P.R. Chen, N.C. Lin, H.T. Huang, T.L. Yang, C.Y. Pai, C.S. Tai, W.L. Chen, C.Y. Huang, C.C. Liu, S.L. Weng, K.W. Liao, W.L. Hsu, H.D. Huang, miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database, Nucleic Acids Res. 44 (2016) D239–247.
[10] L. D'Abundo, E. Callegari, A. Bresin, A. Chillemi, B.K. Elamin, P. Guerriero, X. Huang, E. Saccenti, E. Hussein, F. Casciano, P. Secchiero, G. Zauli, G.A. Calin, G. Russo, L.J. Lee, C.M. Croce, G. Marcucci, S. Sabbioni, F. Malavasi, M. Negrini, Anti-leukemic activity of microRNA-26a in a chronic lymphocytic leukemia mouse model, Oncogene 36 (2017) 6617–6626.
[11] N. Daver, Y. Boumber, H. Kantarjian, F. Ravandi, J. Cortes, M.E. Rytting, J.D. Kawedia, J. Basnett, K.S. Culotta, Z. Zeng, H. Lu, M.A. Richie, R. Garris, L. Xiao, W. Liu, K.A. Baggerly, E. Jabbour, S. O'Brien, J. Burger, L.J. Bendall, D. Thomas, M. Konopleva, A phase I/II study of the mTOR inhibitor everolimus in combination with HyperCVAD chemotherapy in patients with Relapsed/Refractory acute lymphoblastic leukemia, Clin. Cancer Res. 21 (2015) 2704–2714.
[12] D.L. Dong, C. Chen, R. Huo, N. Wang, Z. Li, Y.J. Tu, J.T. Hu, X. Chu, W. Huang, B.F. Yang, Reciprocal repression between microRNA-133 and calcineurin regulates cardiac hypertrophy: a novel mechanism for progressive cardiac hypertrophy, Hypertension 55 (2010) 946–952.
[13] Y. Dong, C. Chang, J. Liu, J. Qiang, Targeting of GIT1 by miR-149* in breast cancer suppresses cell proliferation and metastasis in vitro and tumor growth in vivo, Onco. Ther. 10 (2017) 5873–5882.
[14] D. Duarte, E.D. Hawkins, C. Lo Celso, The interplay of leukemia cells and the bone marrow microenvironment, Blood 131 (2018) 1507–1511.
[15] M.L. Dustin, R. Rothlein, A.K. Bhan, C.A. Dinarello, T.A. Springer, Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1), J. Immunol. 137 (1986) 245–254 J. Immunol. 186 (2011) 5024–5033.
[16] E.P. Erkan, X.O. Breakefield, O. Saydam, miRNA signature of schwannomas: possible role(s) of "tumor suppressor" miRNAs in benign tumors, Oncotarget 2 (2011) 265–270.
[17] Q. Fernandes, MicroRNA: defining a new niche in leukemia, Blood Rev. 31 (2017) 129–138.
[18] A. Flores-Perez, L.A. Marchat, S. Rodriguez-Cuevas, V.P. Bautista, L. Fuentes-Mera, D. Romero-Zamora, A. Maciel-Dominguez, O.H. de la Cruz, M. Fonseca-Sanchez, E. Ruiz-Garcia, H.A. la Vega, C. Lopez-Camarillo, Suppression of cell migration is promoted by miR-944 through targeting of SIAH1 and PTP4A1 in breast cancer cells, BMC Cancer 16 (2016) 379.
[19] B. Fromm, T. Billipp, L.E. Peck, M. Johansen, J.E. Tarver, B.L. King, J.M. Newcomb, L.F. Sempere, K. Flatmark, E. Hovig, K.J. Peterson, A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome, Annu. Rev. Genet. 49 (2015) 213–242.
[20] S. Ganesan, V. Lakshmanan, H.K. Palani, N. Balasundaram, A.A. Alex, S. David, A. Korula, B. George, P. Balasubramanian, H. Gowda, N. Vyas, D. Palakodeti, V. Mathews, Role of miRNA in micro-environment mediated drug resistance in acute promyelocytic leukemia to arsenic trioxide, Blood 128 (2016).
[21] L.A. Gilbert, M.T. Hemann, DNA damage-mediated induction of a chemoresistant niche, Cell 143 (2010) 355–366.
[22] W. Huang da, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, Nucleic Acids Res. 37 (2009) 1–13.
[23] G. Iyer, A.J. Hanrahan, M.I. Milowsky, H. Al-Ahmadie, S.N. Scott, M. Janakiraman, M. Pirun, C. Sander, N.D. Socci, I. Ostrovnaya, A. Viale, A. Heguy, L. Peng, T.A. Chan, B. Bochner, D.F. Bajorin, M.F. Berger, B.S. Taylor, D.B. Solit, Genome sequencing identifies a basis for everolimus sensitivity, Science 338 (2012) 221.
[24] V. Kumar, R. Palermo, C. Talora, A.F. Campese, S. Checquolo, D. Bellavia, L. Tottone, G. Testa, E. Miele, S. Indraccolo, A. Amadori, E. Ferretti, A. Gulino, A. Vacca, I. Screpanti, Notch and NF-kB signaling pathways regulate miR-223/FBXW7 axis in T-cell acute lymphoblastic leukemia, Leukemia 28 (2014) 2324–2335.
[25] H. Li, X. Kong, G. Cui, C. Ren, S. Fan, L. Sun, Y. Zhang, R. Cao, Y. Li, J. Zhou, Rapamycin restores p14, p15 and p57 expression and inhibits the mTOR/p70S6K pathway in acute lymphoblastic leukemia cells, Int. J. Hematol. 102 (2015) 558–568.
[26] R.J. Lin, Y.C. Lin, A.L. Yu, miR-149* induces apoptosis by inhibiting Akt1 and E2F1 in human cancer cells, Mol. Carcinog. 49 (2010) 719–727.
[27] S. Liu, L. Song, H. Yao, L. Zhang, D. Xu, Q. Li, Y. Li, Preserved miR-361-3p expression is an independent prognostic Indicator of favorable survival in cervical cancer, Dis. Markers 2018 (2018) 8949606.
[28] P. Loher, I. Rigoutsos, Interactive exploration of RNA22 microRNA target predictions, Bioinformatics 28 (2012) 3322–3323.
[29] L. Lv, X. Wang, T. Ma, microRNA-944 inhibits the malignancy of hepatocellular carcinoma by directly targeting IGF-1R and deactivating the PI3K/Akt signaling pathway, Cancer Manag. Res. 11 (2019) 2531–2543.
[30] A. Markovic, K.L. MacKenzie, R.B. Lock, Induction of vascular endothelial growth factor secretion by childhood acute lymphoblastic leukemia cells via the FLT-3 signaling pathway, Mol. Cancer Ther. 11 (2012) 183–193.
[31] M. Marz, S. Meyer, U. Erb, C. Georgikou, M.A. Horstmann, S. Hetjens, C. Weiss, P. Fallier-Becker, E. Vandenhaute, H. Ishikawa, H. Schroten, M. Durken, M. Karremann, Pediatric acute lymphoblastic leukemia-conquering the CNS across the choroid plexus, Leuk. Res. 71 (2018) 47–54.
[32] K.J. Mavrakis, J. Van Der Meulen, A.L. Wolfe, X. Liu, E. Mets, T. Taghon, A.A. Khan, M. Setty, P. Rondou, P. Vandenberghe, E. Delabesse, Y. Benoit, N.B. Socci, C.S. Leslie, P. Van Vlierberghe, F. Speleman, H.G. Wendel, A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL), Nat. Genet. 43 (2011) 673–678.
[33] K. Mitra, A.R. Carvunis, S.K. Ramesh, T. Ideker, Integrative approaches for finding modular structure in biological networks, Nat. Rev. Genet. 14 (2013) 719–732.
[34] J.W. Nam, O.S. Rissland, D. Koppstein, C. Abreu-Goodger, C.H. Jan, V. Agarwal, M.A. Yildirim, A. Rodriguez, D.P. Bartel, Global analyses of the effect of different cellular contexts on microRNA targeting, Mol. Cell 53 (2014) 1031–1043.
[35] S.R. Oruganti, D.J. Torres, S. Krebsbach, F. Asperti-Boursin, J. Winters, K. Matlawska-Wasowska, S.S. Winter, C. Halsey, J.L. Cannon, CARMA1 is a novel regulator of T-ALL disease and leukemic cell migration to the CNS, Leukemia 31 (2017) 255–258.
[36] T. Pan, W. Chen, X. Yuan, J. Shen, C. Qin, L. Wang, miR-944 inhibits metastasis of gastric cancer by preventing the epithelial-mesenchymal transition via MACC1/Met/AKT signaling, FEBS Open Bio 7 (2017) 905–914.
[37] M.D. Paraskevopoulou, G. Georgakilas, N. Kostoulas, I.S. Vlachos, T. Vergoulis, M. Reczko, C. Filippidis, T. Dalamagas, A.G. Hatzigeorgiou, DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows, Nucleic Acids Res. 41 (2013) W169–173.
[38] A.E. Place, Y. Pikman, K.E. Stevenson, M.H. Harris, M. Pauly, M.L. Sulis, N. Hijiya, L. Gore, T.M. Cooper, M.L. Loh, G. Roti, D.S. Neuberg, S.K. Hunt, S. Orloff-Parry, K. Stegmaier, S.E. Sallan, L.B. Silverman, Phase I trial of the mTOR inhibitor everolimus in combination with multi-agent chemotherapy in relapsed childhood acute lymphoblastic leukemia, Pediatr. Blood Cancer 65 (2018) e27062.
[39] K. Podshivalova, E.A. Wang, T. Hart, D.R. Salomon, Expression of the miR-150 tumor suppressor is restored by and synergizes with rapamycin in a human leukemia T-cell line, Leuk. Res. 74 (2018) 1–9.
[40] C.H. Pui, M.V. Relling, J.R. Downing, Acute lymphoblastic leukemia, N. Engl. J. Med. 350 (2004) 1535–1548.
[41] C.H. Pui, E. Thiel, Central nervous system disease in hematologic malignancies: historical perspective and practical applications, Semin. Oncol. 36 (2009) S2–S16.
[42] S.R. Rheingold, S.K. Tasian, J.A. Whitlock, D.T. Teachey, M.J. Borowitz, X. Liu, C.G. Minard, E. Fox, B.J. Weigel, S.M. Blaney, A phase 1 trial of temsirolimus and intensive re-induction chemotherapy for 2nd or greater relapse of acute lymphoblastic leukaemia: a Children's Oncology Group study (ADVL1114), Br. J. Haematol. 177 (2017) 467–474.
[43] C. Roth, I. Stuckrath, K. Pantel, J.R. Izbicki, M. Tachezy, H. Schwarzenbach, Low levels of cell-free circulating miR-361-3p and miR-625* as blood-based markers for discriminating malignant from benign lung tumors, PLoS One 7 (2012) e38248.
[44] V.R. Sanghvi, K.J. Mavrakis, J. Van der Meulen, M. Boice, A.L. Wolfe, M. Carty, P. Mohan, P. Rondou, N.D. Socci, Y. Benoit, T. Taghon, P. Van Vlierberghe, C.S. Leslie, F. Speleman, H.G. Wendel, Characterization of a set of tumor suppressor microRNAs in T cell acute lymphoblastic leukemia, Sci. Signal. 7 (2014) ra111.
[45] D. Schotte, R.X. De Menezes, F. Akbari Moqadam, L.M. Khankahdani, E. Lange-Turenhout, C. Chen, R. Pieters, M.L. Den Boer, MicroRNA characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia, Haematologica 96 (2011) 703–711.
[46] D. Schraivogel, L. Weinmann, D. Beier, G. Tabatabai, A. Eichner, J.Y. Zhu, M. Anton, M. Sixt, M. Weller, C.P. Beier, G. Meister, CAMTA1 is a novel tumour suppressor regulated by miR-9/9* in glioblastoma stem cells, EMBO J. 30 (2011) 4309–4322.
[47] A. Schuermann, C.S. Helker, W. Herzog, Metallothionein 2 regulates endothelial cell migration through transcriptional regulation of vegfc expression, Angiogenesis 18 (2015) 463–475.
[48] M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, N. Rajewsky, Widespread changes in protein synthesis induced by microRNAs, Nature 455 (2008) 58–63.
[49] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, T. Ideker, Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504.

[50] L. Si, L. Xu, L. Yin, Y. Qi, X. Han, Y. Xu, Y. Zhao, K. Liu, J. Peng, Potent effects of dioscin against pancreatic cancer via miR-149-3P-mediated inhibition of the Akt1 signalling pathway, Br. J. Pharmacol. 174 (2017) 553–568.
[51] D. Szklarczyk, A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K.P. Tsafou, M. Kuhn, P. Bork, L.J. Jensen, C. von Mering, STRING v10: protein-protein interaction networks, integrated over the tree of life, Nucleic Acids Res. 43 (2015) D447–452.
[52] N. Wagle, B.C. Grabiner, E.M. Van Allen, E. Hodis, S. Jacobus, J.G. Supko, M. Stewart, T.K. Choueiri, L. Gandhi, J.M. Cleary, A.A. Elfiky, M.E. Taplin, E.C. Stack, S. Signoretti, M. Loda, G.I. Shapiro, D.M. Sabatini, E.S. Lander, S.B. Gabriel, P.W. Kantoff, L.A. Garraway, J.E. Rosenberg, Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib, Cancer Discov. 4 (2014) 546–553.
[53] L. Wen, Y. Li, Z. Jiang, Y. Zhang, B. Yang, F. Han, miR-944 inhibits cell migration and invasion by targeting MACC1 in colorectal cancer, Oncol. Rep. 37 (2017) 3415–3422.
[54] N. Wong, X. Wang, miRDB: an online resource for microRNA target prediction and functional annotations, Nucleic Acids Res. 43 (2015) D146–152.
[55] D. Yang, G. Du, A. Xu, X. Xi, D. Li, Expression of miR-149-3p inhibits proliferation, migration, and invasion of bladder cancer by targeting S100A4, Am. J. Cancer Res. 7 (2017) 2209–2219.
[56] Y.Y. Ye, J.W. Mei, S.S. Xiang, H.F. Li, Q. Ma, X.L. Song, Z. Wang, Y.C. Zhang, Y.C. Liu, Y.P. Jin, Y.P. Hu, L. Jiang, F.T. Liu, Y.J. Zhang, Y.J. Hao, Y.B. Liu, MicroRNA-30a-5p inhibits gallbladder cancer cell proliferation, migration and metastasis by targeting E2F7, Cell Death Dis. 9 (2018) 410.
[57] Y. Zhang, J. Hou, F. Ge, F. Cao, H. Li, P. Wang, M. Xu, P. Song, X. Li, S. Wang, J. Li, X. Han, Y. Zhao, Y. Su, Y. Li, S. Fan, L. Li, J. Zhou, Integrating microRNA and mRNA expression profiles of acute promyelocytic leukemia cells to explore the occurrence mechanisms of differentiation syndrome, Oncotarget 7 (2016) 73509–73524.
[58] Y. Zhao, J. Ma, Y. Fan, Z. Wang, R. Tian, W. Ji, F. Zhang, R. Niu, TGF-beta transactivates EGFR and facilitates breast cancer migration and invasion through canonical Smad3 and ERK/Sp1 signaling pathways, Mol. Oncol. 12 (2018) 305–321.
[59] C.M. Zwaan, J. Kowalczyk, C. Schmitt, B. Bielorai, M.W. Russo, M. Woessner, S. Ranganathan, G. Leverger, Safety and efficacy of nelarabine in children and young adults with relapsed or refractory T-lineage acute lymphoblastic leukaemia or T-lineage lymphoblastic lymphoma: results of a phase 4 study, Br. J. Haematol. 179 (2017) 284–293.