Pathol. Oncol. Res. (2015) 21:875–880
DOI 10.1007/s12253-015-9902-2

RESEARCH

Identification of Target Genes Regulated by KSHV miRNAs in KSHV-Infected Lymphoma Cells

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Received: 12 August 2014 / Accepted: 9 January 2015 / Published online: 4 February 2015

Abstract This study aimed to identify target genes regulated by KSHV miRNAs in KSHV-infected lymphoma cells. Original Ago HITS-CLIP data of BC-3 and BCBL-1 cell lines were downloaded from SRA database in NCBI, including mRNA and miRNA samples. The raw mRNA reads were mapped into human reference genome hg19 via TopHat for read alignment. PCR duplicates were removed via the SAM tool and the peaks of reads were analyzed via Cufflinks. For miRNA data, the raw data were mapped to the mature miRNA sequences based on miRBase via Bowtie. Peak intersection was computed by using intersectBed in BEDtools. Then, the mature miRNA seeds were identified and then were aligned with 3' UTR merged peaks. The regulationships of miRNAs and their corresponding genes were analyzed based on the signal of RNA-induced silencing complex. Totally, 7 KSHV-related genes regulated by KSHV miRNAs were identified, including *IPO5*, *EDA*, *NT5C3*, *WSB1*, *KCNS1*, *PRAM1* and *MTRNR2L6*. Among them, *EDA*, *MTRNR2L6* and *IPO5* were regulated by multiple KSHV miRNAs, such as kshv-miR-K12-1-5p, kshv-miR-K12-4-3p and kshv-miR-K12-3-5p, respectively. Furthermore, expression of kshv-miR-K12-1-5p and kshv-miR-K12-3-5p in BCBL-1 cell line were lower than that in BC-3 cell line, conversely, expression of kshv-miR-K12-4-3p in BCBL-1 cell line were higher than that in BC-3 cell line. In conclusion, potential target genes regulated by KSHV miRNAs in KSHV-infected lymphoma cells might play key roles in the nosogenesis of this disease. These findings might provide the basis for deep understanding of KSHV-infected tumors and further molecular experiments.

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Keywords Kaposi's sarcoma-associated herpesvirus · Gene · microRNAs · Functional annotation

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is correlated with primary effusion lymphomas (PEL) [1]. Like other herpesviruses, the KSHV encodes microRNAs (miRNAs) and these miRNAs can highly restrict viral gene expression and be associated with many critical processes in viral life cycle, such as viral latency control, viral immune evasion, host cell cycle arrest and antiapoptosis [2]. To date, some cellular targets have been identified for the KSHV miRNAs in PEL cell lines. For instance, *BACH1* (basic leucine zipper transcription factor 1), a transcriptional repressor, has been suggested as a target of kshv-miR-K12-11 (homology to cellular miR-155) in KSHV-infected BCBL-1 cells [3]. *MAF* (Musculoaponeurotic fibrosarcoma oncogene homolog), an endothelial cell-specific transcription factor, has been found to be targeted by kshv-miR-K12-11 and kshv-miR-K12-6-5p in KSHV-infected lymphatic endothelial cells (LECs) [4]. Besides, in BC-3 cells, miR-K1 attenuates p21-mediated cell cycle arrest by repressing p21 [5].

Argonaute (Ago) proteins are the catalytic components of the RNA-induced silencing complex (RISC) and can bind miRNAs. In 2012, Haecker et al. have identified numerous KSHV miRNAs and their targets in two latently KSHV-infected primary effusion lymphoma (PEL) cell lines BCBL-1 and BC-3, respectively, using Ago HITS-CLIP (High-throughput sequencing of cross-linking immunoprecipitation). They have found that enriched clusters contained KSHV miRNA seed matches in the 3' UTRs of numerous well characterized targets, among them *THBS1*, *BACH1*, and *C/EBPb*.

However, more targets of KSHV miRNAs need to be identified using bioinformatics method. In this study, raw Ago HITS-CLIP data of BCBL-1 and BC-3 cell lines deposited by Haecker et al. [6] were download from website. By using the online database and bioinformatics software, the cellar genes related to KSHV have been identified. Then functional annotation of genes and the potential KSHV miRNAs targeting those genes related to KSHV were analyzed. All these studies might contribute to the better understanding of molecular mechanisms of KSHV-infected lymphoma cells.

Materials and Methods

Raw Ago HITS-CLIP Data of Sarcoma

The raw profiling data SRP016003 of two KSHV-infected primary effusion lymphoma (PEL) cell lines (BCBL-1 and BC-3) [6] were obtained from Short Read Archive (SRA) database in National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), which was based on the platform GPL10999 Illumina Genome Analyzer IIx (Homo sapiens) (Illumina, CA, USA). The data of BC-3 cell lines contained 3 mRNA samples under access number SRR580356, SRR580357 and SRR580358 and 3 miRNA samples under access number SRR580359, SRR580360 and SRR580361. Meanwhile, the data of BCBL-1 cell lines included 4 mRNA expression samples under access number SRR580352, SRR580353, SRR580354 and SRR580355 and 2 miRNA samples under access number SRR580362 and SRR580363. The high-throughput sequencing.

Read Alignment and Peak Analysis of mRNA and miRNA Data

The mRNA expression profiling data from BC-3 and BCBL-1 cell lines were mapped to the reference human genome (hg19) of University of California Santa Cruz (UCSC) by using TopHat software [7] for read alignment. The best alignment of each read was selected. After that, PCR duplicates were removed via the Sequence Alignment/Map (SAM) tool [8]. Cufflinks [9] software was used to analyze the peaks of reads. The peak length threshold<25 % of total peak length and the peak coverage threshold>75 % of total peak depth were set as the cut-off criteria.

Meanwhile, the miRNA expression profiling data from BC-3 and BCBL-1 cell lines were aligned with the known mature miRNA sequences of human KSHV which were selected from miRBase (http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2010), using Bowtie software [10]. A maximum of one mismatch and the unique genomic location mapping of each read were required.

For peaks shared by every sample, peak intersection was computed by using intersectBed in Bedtools [11]. The peak overlapping between two samples>90 % was set as the cut-off criterion. Then, peaks were merged via BEDtools [11]. According to the peak annotation, the merged peak located in 3' UTR of gene (3' UTR merged peak) was utilized for further study.

Processing of miRNA Data and miRNA Seed

The seed region (nucleotides 2–9) of the known mature miRNA sequences of KSHV was identified and then was aligned with 3' UTR merged peak. A maximum of one mismatch and the unique location mapping of each read were required. Given the complementarity between miRNA seed sequences and mRNA 3' UTR sequences, the KSHV miRNAs whose seeds matched with 3' UTR of protein coding genes were screened out.

GO Functional Annotation

The Database for Annotation, Visualization and Integrated Discovery (DAVID) [12] was used to perform GO functional annotation [13] for genes. GO categories were classified into Biological Process (BP) GO-terms, Molecular Function (MF) GO-terms and Cellular Component (CC) GO-terms.

Detection of KSHV miRNAs Expression Levels

To further confirm the potential KSHV miRNAs modulating KSHV-related genes, the expression levels of KSHV miRNAs in RISC complex in the two cell lines were detected.

Results

Identification of 3' UTR Merged Peak

A total of 434,240 and 135,256 reads respectively in BC-3 and BCBL-1 cell lines were obtained from SRA database. After read alignment and peak analysis, 1,941 and 299 peaks were respectively computed, indicating that mRNA regulation mediated by RNA-induced silencing complex (RISC) complex in BCBL-1 cell line was less active than that in BC-3 cell line. Furthermore, total 1,102 (57 %) and 189 (63 %) 3' UTR merged peaks were identified from BC-3 and BCBL-1 cell lines, respectively. Other peaks were located in intron, exon or intergenic region. The detail information was shown in Table 1.

Table 1 Preprocessing results of raw Ago HITS-CLIP data of BC-3 and BCBL-1 cells

Category	Count in BC-3	Count in BCBL-1
Total reads	60770019	15638325
Mappable reads	49054353	11054175
Remove PCR duplicates	434240	135256
Total peaks	3452	711
Peaks filtering	1941	299
3'UTR merged peak	1102	189

Ago HITS-CLIP Argonaute high-throughput sequencing of cross-linking immunoprecipitation

Functional Annotation of Genes Related to KSHV

To confirm the miRNA-gene abnormal-regulation associated with KSHV, 102 peaks shared by two cell lines were identified, 7 of which were located in the 3' UTR region of protein coding genes, including *IPO5* (importin 5), *EDA* (ectodysplasin A), *NT5C3* (5'-nucleotidase, cytosolic III), *WSB1* (WD repeat and SOCS box-containing 1), *KCNS1* (potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1), *PRAM1* (PML-RARA regulated adaptor molecule 1) and *MTRNR2L6* (humanin-like protein 6) (Table 2).

Go functional annotation of the 7 potential genes showed that *KCNS1* and *NT5C3* were mainly related to metal ion binding. However, *KCNS1* and *NT5C3* were located in plasma membrane part and endoplasmic reticulum, respectively. *IPO5*, located in nucleolus, might be associated with protein transporter activity. *WSB1* was involved in modification-dependent macromolecule catabolic process, whereas the cellular localization was undefined. Besides, *PRAM1* and *EDA* both presented in the regulation of cellular localization. Notably, *EDA* showed the function of tumor necrosis factor receptor binding and played a role in the positive regulation of NF-kappa B transcription factor activity (Table 3).

Table 2 Genes related to KSHV

RefSeq ID	Symbol	Description
NM_002271	*IPO5*	importin 5
NM_001399	*EDA*	ectodysplasin A
NM_001166118	*NT5C3*	5'-nucleotidase, cytosolic III
NM_134265	*WSB1*	WD repeat and SOCS box-containing 1
NM_002251	*KCNS1*	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
NM_032152	*PRAM1*	PML-RARA regulated adaptor molecule 1
NM_001190487	*MTRNR2L6*	humanin-like protein 6

KSHV Kaposi's sarcoma-associated herpesvirus

Potential KSHV miRNAs Targeting the Seven Genes

EDA, *MTRNR2L6* and *IPO5* were regulated by 7, 6 and 6 KSHV miRNAs, respectively, whereas *KCNS*, *NT5C3*, *WSB1* and *PRAM1* were separately regulated by 2, 1, 2 and 1 KSHV miRNAs, suggesting that *EDA*, *MTRNR2L6* and *IPO5* might be the main targets regulated by KSHV miRNAs (Table 4).

Analysis of KSHV miRNAs the RISC Complex in the Two Cell Lines

Among the above 25 known KSHV miRNAs, kshv-miR-K12-9-5p, kshv-miR-K12-8-5p, kshv-miR-K12-3-3p, kshv-miR-K12-4-3p, kshv-miR-K12-1-5p and kshv-miR-K12-3-5p could form the RISC complex in BC-3 and BCBL-1 cell lines.

Briefly, the expression level of kshv-miR-K12-3-3p in BCBL-1 cell line was higher than that in BC-3 cell line, as well as the kshv-miR-K12-4-3p. Meanwhile, the expression

Table 3 Gene ontology (GO) function annotation of potential genes related to KSHV

Category	Term	Count	Genes
BP	GO:0060341 regulation of cellular localization	2	*PRAM1*, *EDA*
BP	GO:0051092 positive regulation of NF-kappa B transcription factor activity	1	*EDA*
BP	GO:0055085 transmembrane transport	1	*KCNS1*
BP	GO:0017038 protein import	1	*IPO5*
BP	GO:0043632 modification-dependent macromolecule catabolic process	1	*WSB1*
CC	GO:0005783 endoplasmic reticulum	2	*NT5C3*, *EDA*
CC	GO:0044459 plasma membrane part	2	*KCNS1*, *EDA*
CC	GO:0005576 extracellular region	1	*EDA*
CC	GO:0005730 nucleolus	1	*IPO5*
CC	GO:0008076 voltage-gated potassium channel complex	1	*KCNS1*
MF	GO:0046872 metal ion binding	2	*KCNS1*, *NT5C3*
MF	GO:0008565 protein transporter activity	1	*IPO5*
MF	GO:0008289 lipid binding	1	*PRAM1*
MF	GO:0005164 tumor necrosis factor receptor binding	1	*EDA*

KSHV Kaposi's sarcoma-associated herpesvirus, *BP* biological process, *CC* cellular component, *MF* molecular function

Table 4 Potential microRNAs for genes related to KSHV

Gene symbol	microRNA count	microRNAs
KCNS	2	kshv-miR-K12-10a-5p kshv-miR-K12-12-3p
IPO5	6	kshv-miR-K12-10a-3p, kshv-miR-K12-11-5p, kshv-miR-K12-2-5p, kshv-miR-K12-2-3p, kshv-miR-K12-9-3p, kshv-miR-K12-3-5p
EDA	7	kshv-miR-K12-10b, kshv-miR-K12-1-5p, kshv-miR-K12-1-3p, kshv-miR-K12-8-3p, kshv-miR-K12-5-5p, kshv-miR-K12-12-5p, kshv-miR-K12-6-5p
MTRNR2L6	6	kshv-miR-K12-11-3p, kshv-miR-K12-7-5p, kshv-miR-K12-5-3p, kshv-miR-K12-4-5p, kshv-miR-K12-4-3p kshv-miR-K12-7-3p
NT5C3	1	kshv-miR-K12-9-5p
WSB1	2	kshv-miR-K12-8-5p, kshv-miR-K12-6-3p
PRAM1	1	kshv-miR-K12-3-3p

KSHV Kaposi's sarcoma-associated herpesvirus

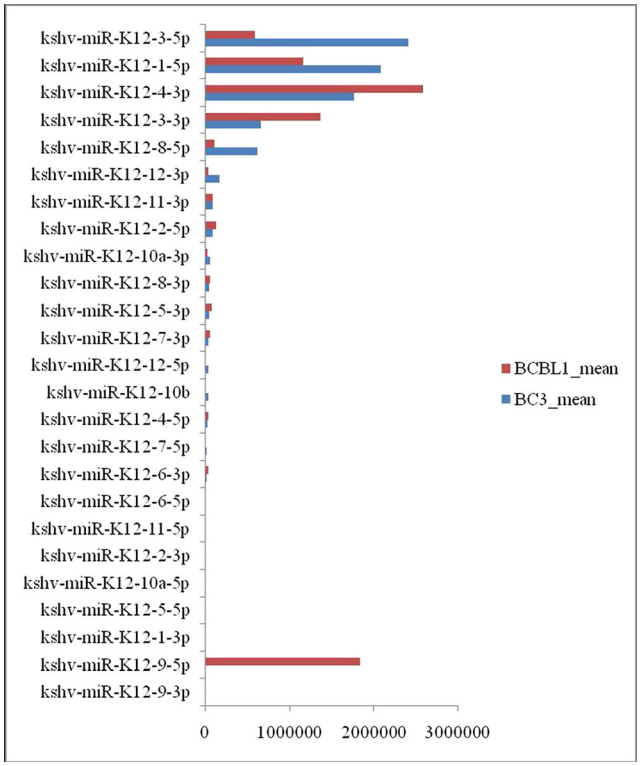

Fig. 1 The expression levels of KSHV miRNAs in BC-3 and BCBL cell lines.

levels of kshv-miR-K12-8-5p, kshv-miR-K12-1-5p and kshv-miR-K12-3-5p in BCBL-1 cell line were lower than that in BC-3 cell line. Notably, kshv-miR-K12-9-5p was only expressed in BCBL-1 cell line (Fig. 1).

In summary, in the BC-3 cell line, the RISC complexes formed by kshv-miR-K12-1-5p, kshv-miR-K12-4-3p and kshv-miR-K12-3-5p were combined with 3' UTR region of *EDA*, *MTRNR2L6* and *IPO5*, respectively (Fig. 2). In the BCBL-1 cell line, the regulation between kshv-miR-K12-1-5p and *EDA* were also detected, as well as regulation between kshv-miR-K12-4-3p and *MTRNR2L6*; however, the ability of kshv-miR-K12-3-5p to regulate *IPO5* was lower than that in BC-3 cell line. Besides, the regulations between kshv-miR-K12-9-5p and *NT5C3*, as well as kshv-miR-K12-3-3p and *PRAM1* were detected in BCBL-1 cell line (Fig. 3).

Discussion

In the present study, total 7 potential target genes of KSHV miRNAs were identified, including *IPO5*, *EDA*, *NT5C3*, *WSB1*, *KCNS1*, *PRAM1* and *MTRNR2L6*. Among them, *EDA*, *IPO5* and *MTRNR2L6* were regulated by multiple KSHV miRNAs.

Ectodysplasin-A (EDA), a novel ligand of the tumor necrosis factor (TNF) family, controls ectodermal differentiation, of which EDA-A1 and EDA-A2 are the two major isoforms binding to ectodermal dysplasia receptor (EDAR) and X-linked EDAR (XEDAR), respectively [14]. EDA was found to be annotated in positive regulation of NF-kappaB transcription factor activity and tumor necrosis factor receptor binding in our study. Study has shown that the transcription factor NF-κB can be constitutively activated by KSHV in lymphomas, which is necessary for survival of KSHV-infected lymphoma cells [15] and the development of malignant neoplasia [16]. Besides, TNF receptor can activate NF-κB [17]. *EDA* was regulated by kshv-miR-K12-1-5p in both BC-3 and BCBL-1 cell lines. It has been reported that the KSHV-encoded miRNA, miR-K12-1-5p, directly targets the transcript of host gene IκBα, which modulates the NF-κB pathway and reduces lytic activation [18]. Therefore, *EDA* might play a pivotal role in KSHV-infected lymphoma cells, via the regulation of kshv-miR-K12-1-5p.

In our study, *MTRNR2L6* (MT-RNR2-like 6) was discovered to be regulated by 6 KSHV miRNAs, such as kshv-miR-K12-4-3p and kshv-miR-K12-11-3p. *MTRNR2L6* encodes humanin (HN)-6 (HN6) [19]. Humanin (HN) is a neuroprotective and antiapoptotic peptide derived from the mitochondrial *MTRNR2*-like gene [20]. HN inhibits apoptosis via interaction with the BCL2/BAX proapoptotic protein family, such as

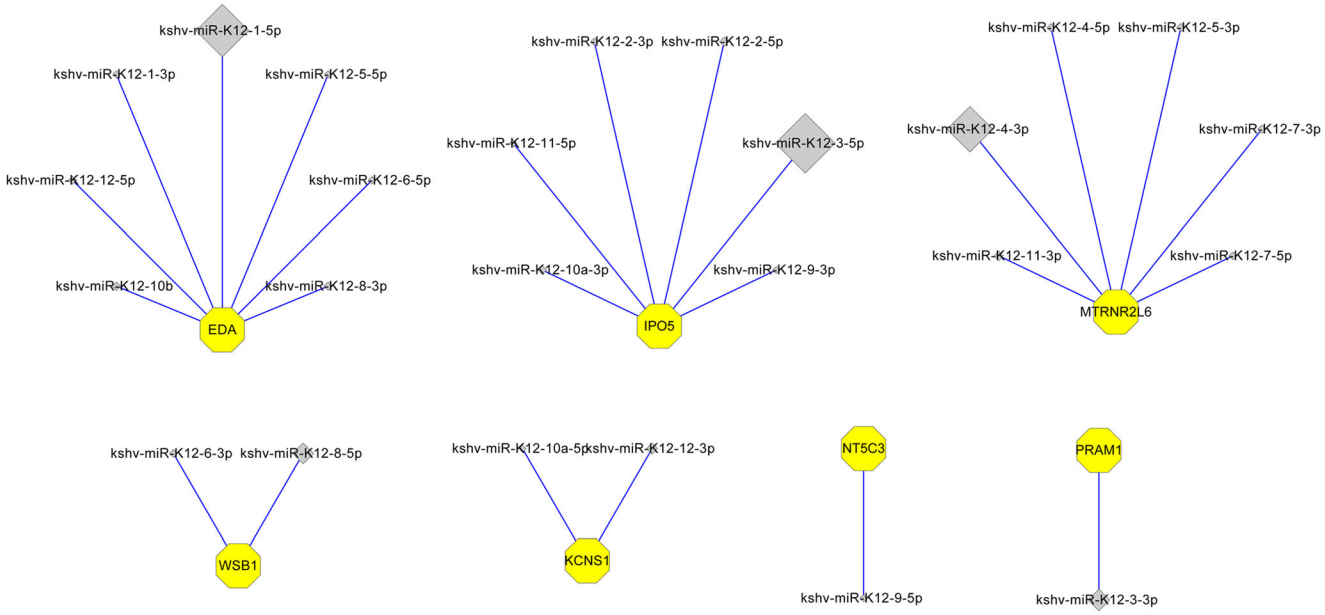

Fig. 2 The regulationship display of KSHV miRNAs and their target gene in BC-3 cell line. *Gray diamond* represents KSHV miRNAs; *Yellow octagon* represents the KSHV-related genes. The size of the diamond is proportional to the signal level of KSHV miRNAs. The *lines* represent the regulation between KSHV miRNAs and KSHV-related genes.

BAX, BID and BCL2L11 [21, 22]. *BCL2L11* is one of the kshv-miR-K12-11-responsive genes known to be aberrantly expressed in human malignancies [23]. However, the study on *MTRNR2L6* in KSHV-infected lymphoma cells was limited. Therefore, *MTRNR2L6* was speculated to be crucial in KSHV-infected lymphoma cells through the regulation of kshv-miR-K12-4-3p and kshv-miR-K12-11-3p.

IPO5 (importin 5) was also found to be regulated by 6 KSHV miRNAs, such as kshv-miR-K12-3-5p. *IPO5* was mainly associated with protein transport. During the nucleocytoplasmic transport, the import of proteins requires the nuclear localization signal (NLS) import receptor, a heterodimer of importin alpha and beta subunits [24]. Protein transport is an indispensable process during the infection of KSHV to lymphoma cells. Hence, *IPO5* might play a key role

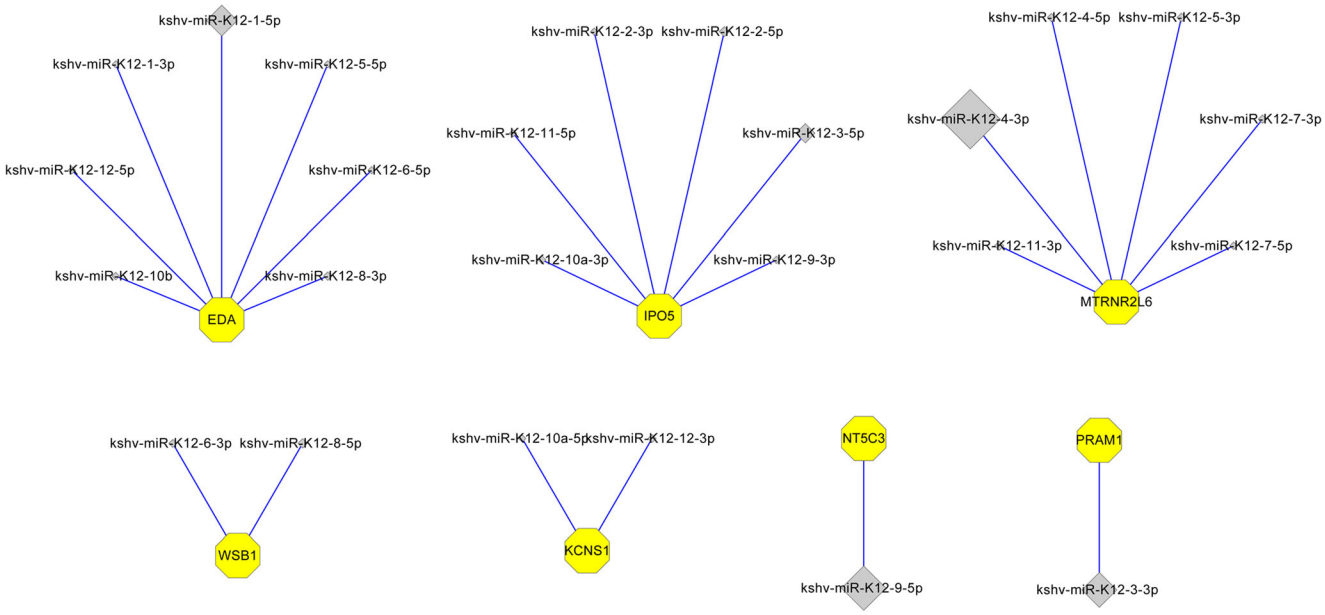

Fig. 3 The regulation of KSHV miRNAs in BCBL-1 cell line. *Gray diamond* represents KSHV miRNAs; *Yellow octagon* represents the KSHV-related genes. The size of the diamond is proportional to the signal level of KSHV miRNAs. The *lines* represent the regulation between KSHV miRNAs and KSHV-related genes.

in the protein transport in KSHV-infected lymphoma cells, by the regulation of multiple miRNAs, such as kshv-miR-K12-3-5p.

However, more molecular experiments were needed to verify our hypothesis. The main important research in the future may be focused on the functional consequences of KSHV miRNAs and their regulation in cells.

In conclusion, a total of 7 KSHV-related genes regulated by KSHV miRNAs were identified. High expression of kshv-miR-K12-1-5p, kshv-miR-K12-4-3p and kshv-miR-K12-3-5p might inhibit the translation of *EDA*, *MTRNR2L6* and *IPO5*, respectively. These results might improve the deep understanding of the pathogenesis of KSHV-infected lymphoma cells, and provide a theoretical foundation for further experimental studies.

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