

RESEARCH REPORT

Prediction of human targets for viral-encoded microRNAs by thermodynamics and empirical constraints

Alessandro Laganà^{1,2,*}, Stefano Forte³, Francesco Russo², Rosalba Giugno^{1,§}, Alfredo Pulvirenti^{1,§}, Alfredo Ferro^{1,2}

¹Department of Mathematics and Computer Science, University of Catania, Italy, ²Department of Biomedical Sciences, University of Catania, Italy, ³IOM Ricerca, Viagrande, Italy

*Correspondence to: Alessandro Laganà, Email: lagana@dmi.unict.it, Tel: +39 095 7337208, Fax: +39 095 330094

§These authors contributed equally to this work.

Received 26 February 2010; Revised 03 May 2010; Accepted 17 May 2010; Published online 24 May 2010

J RNAi Gene Silencing, 2010, 6(1), 379-385

© Copyright The Authors: This is an open access article, published under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.0/uk/>). This license permits non-commercial use, distribution and reproduction of the article, provided the original work is appropriately acknowledged with correct citation details.

ABSTRACT

MicroRNAs (miRNAs) are small RNA molecules that modulate gene expression through degradation of specific mRNAs and/or repression of their translation. miRNAs are involved in both physiological and pathological processes, such as apoptosis and cancer. Their presence has been demonstrated in several organisms as well as in viruses. Virus encoded miRNAs can act as viral gene expression regulators, but they may also interfere with the expression of host genes. Viral miRNAs may control host cell proliferation by targeting cell-cycle and apoptosis regulators. Therefore, they could be involved in cancer pathogenesis. Computational prediction of miRNA/target pairs is a fundamental step in these studies. Here, we describe the use of miRiam, a novel program based on both thermodynamics features and empirical constraints, to predict viral miRNAs/human targets interactions. miRiam exploits target mRNA secondary structure accessibility and interaction rules, inferred from validated miRNA/mRNA pairs. A set of genes involved in apoptosis and cell-cycle regulation was identified as target for our studies. This choice was supported by the knowledge that DNA tumor viruses interfere with the above processes in humans. miRNAs were selected from two cancer-related viruses, Epstein-Barr Virus (EBV) and Kaposi-Sarcoma-Associated Herpes Virus (KSHV). Results show that several transcripts possess potential binding sites for these miRNAs. This work has produced a set of plausible hypotheses of involvement of v-miRNAs and human apoptosis genes in cancer development. Our results suggest that during viral infection, besides the protein-based host regulation mechanism, a post-transcriptional level interference may exist. miRiam is freely available for downloading at <http://ferrolab.dmi.unict.it/miriam>.

KEYWORDS: miRNA, virus, cancer, apoptosis, cell cycle, EBV, KSHV

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs involved in post-transcriptional gene silencing (PTGS) that is a highly-conserved mechanism for regulating gene expression. miRNAs inhibit protein synthesis by binding

with the 3'UTR or, rarely, the coding sequence of specific mRNAs (Bartel, 2004).

miRNAs are known to be involved in several physiological processes, such as apoptosis, development and cell differentiation, and their aberrant expression is observed in

several diseases such as cancer, neurodegeneration and cardiac pathologies (Garofalo et al, 2008).

miRNAs are also expressed by viruses (v-miRNAs), which may act as self-regulators. It has been shown that the EBV-encoded miRNA, miR-BART2, down-regulates the viral DNA polymerase BALF5, thus inhibiting transition from latent to lytic viral replication (Barth et al, 2008). v-miRNAs can also interfere with the host cell transcripts (Cullen, 2006). The cellular protein p53, an up-regulated modulator of apoptosis (PUMA), is regulated by the EBV-encoded miR-BART5. The expression of miR-BART5 renders infected miR-BART5-expressing cells less sensitive to pro-apoptotic agents, but apoptosis can be triggered by depleting miR-BART5 or inducing the expression of PUMA. This shows the capability of v-miRNAs to facilitate the establishment of latent infection by promoting host cell survival (Choy et al, 2008).

The silencing of some nodes of the apoptotic pathway would give to the virus an important advantage during its lysogenic phase, since the host cells would not die by cell death triggered from infection and the virus could complete its vital cycle. Many v-miRNAs are located in proximity or inside viral latency genes and, presumably, co-transcribed with them (Nair and Zavolan, 2006). On the other hand, in many cases of viral infection the activation of apoptosis is needed during the lytic cycle of the virus allowing the processing of capsids and viral particles dissemination (Mendez et al, 2004).

The negative and positive modulation of the apoptotic process is a fundamental activity for beginning and concluding the vital cycle of the virus. The above mentioned cases and other recent findings show that, besides well-known protein mechanisms of apoptosis control by viruses, there are other strategies of apoptosis regulation based on translational repression by miRNAs (Klase et al, 2009).

Apoptosis may also represent one of the most important links between viral infections and oncogenesis. All tumor related viruses seem to share the same approach to interfere with apoptosis and cell-cycle genes (Damania, 2007). Thus, they may directly promote neoplastic transformation of infected cells or facilitate cancer insurgence in favourable contexts. For example, the Kaposi Sarcoma Herpes Virus encodes a protein called LANA, which inhibits the tumor suppressor protein p53, thus promoting cell survival, virus latency and oncogenesis (Si and Robertson, 2006).

In this article we present computational predictions of human targets for v-miRNAs encoded by two tumor related viruses, Epstein-Barr Virus (EBV) and Kaposi Sarcoma Herpes Virus (KSHV). The predictions are computed by a novel approach, which combines thermodynamics and empirical constraints in order to produce reliable miRNA/target duplexes.

Our results demonstrate v-miRNA/human target pairs which possess good binding scores, some of which are also supported by publicly available data on gene

expression profiles. These findings are consistent with previously reported regulation mechanisms and offer a set of candidate targets for further biological validation.

MATERIALS AND METHODS

Computational prediction of v-miRNA/target duplexes

v-miRNAs/human target interactions were predicted by miRiam, a novel tool capable of screening large databases of miRNAs in order to identify the most probable binding sites on a given mRNA, in a reasonable time. miRiam is based on a combined approach which makes use of thermodynamics and pairing constraints inferred from known miRNA/target pairs.

The importance of target secondary structure in miRNA-based silencing has been previously demonstrated (Robins et al, 2005). Target binding sites must be accessible and interactions with miRNAs have to be energetically favourable (Kertesz et al, 2007). Moreover, to predict duplexes reliably several features inferred from known miRNA/mRNA pairs need to be taken into account. For example, miRNA 5' end contains the seed region, which is important in their binding with the target using near perfect complementarity. Nevertheless, the seed region may sometimes contain G:U wobbles, and the number of G:U wobbles in this region appears to be inversely related to silencing efficacy of the miRNA. Almost all known miRNA binding sites are located in the 3' UTRs of target mRNAs, and multiple binding sites have been observed in several cases, potentially increasing the efficacy of silencing (Grimson et al, 2007).

Given a target mRNA sequence and a database of miRNAs, which can be retrieved from miRBase, miRiam performs the following steps:

i) Target accessibility evaluation: Base pairing probabilities of the target are computed by Vienna RNA package functions (Hofacker et al, 1994) and used to identify accessible regions, *i.e.* less paired sub-sequences.

ii) Alignment of miRNA/binding sites: The complementary base-pair alignment of accessible sub-sequences with miRNAs is then computed. A variant of the classical Needleman-Wunsch pairwise sequence alignment in which only matches of complementary base pairs are allowed is used.

iii) Post alignment filtering: All duplexes which do not satisfy previously described empirical sequence constraints are discarded. The remaining pairs are presented to the user ordered by their binding energies.

miRNAs and targets selection

EBV and KSHV miRNAs were obtained from miRBase (Griffiths-Jones et al, 2008). Target mRNA transcripts were downloaded from GenBank, chosen based on their Gene Ontology annotations. Genes annotated with the following terms were selected: Pro-apoptosis, cell cycle arrest, cell cycle checkpoint, negative regulation of cell cycle, negative regulation of cyclin-dependent kinase activity, and negative regulation of mitotic cell cycle.

Expression data

The expression profiles of the predicted targets were retrieved from the NCBI GEO Profiles database. Among the available datasets, NCBI GDS940 and GDS989 were selected as they were obtained from suitable biological specimens (infected cells and corresponding uninfected controls). For each predicted target, experiments performed on EBV and KSHV infected cells were selected and the ratios between the mean gene expression values in the infected and in the control samples were computed. The evaluation of fold change significance using formal statistical methods was not possible due to the small number of available samples. For this reason the observed ratios should be considered as qualitative descriptors supporting or confuting the proposed hypotheses. The data on the v-miRNA expressing tissues were obtained from the literature (Pfeffer et al, 2004; Pfeffer et al, 2005; Cai and Cullen, 2006; Cai et al, 2006; Kim et al, 2007; Marshall et al, 2007; Zhu et al, 2009) and from the miR-Z database (Hausser et al, 2009).

RESULTS

Representative results of our predictions are shown in Tables 1 and 2. Table 1 contains the EBV miRNAs

predicted targets while Table 2 shows the predictions for KSHV miRNAs. For each v-miRNA, the tissues where it is expressed, the predicted human targets and. Only the transcripts with perfect matches with the seed of v-miRNAs in structurally-accessible regions were selected (maximum one G:U wobble allowed in the seed region). These results indicate the putative capability of EBV and KSHV to interfere with the expression of host genes involved in apoptosis and cell cycle regulation. Moreover, some of the predictions are supported by gene expression profiles in EBV and KSHV infected cells.

Table 3 shows, for each predicted target, the ratios between the mean expression values in the infected and control cells. A value less than 1 indicates that the gene is under-expressed in the infected samples, compared to the control cells. Only these values are reported, since they are the significant ones concerning our analysis. The complete data are given in supplementary tables (Tables S1-S3).

Finally, in order to assess the reliability of the proposed method, we performed a comparison between miRiam and some of the most popular available tools for miRNA target

Table 1. Predicted targets for EBV v-miRNAs. For each miRNA, the list of tissues where it is expressed, the predicted targets, the number of site on each target transcript and the associated ontological term are given. Tissues legend: AML: Acute myeloid leukemia, BCLL: B-Chronic lymphocytic leukemia, BL: Burkitt's lymphoma, ESC: Embryonic stem cells, GC: Gastric carcinoma, HD: Hodgkin's disease, L: Lymphoblasts, LAC: Lung's adenocarcinoma, LBCL: Large B-cell lymphoma, LU: Lung, MCL: Mantle cell lymphoma, M: Myeloblasts, NC: Nasopharyngeal carcinoma, PEL: Primary-effusion lymphoma, USSC: Unrestricted somatic stem cells.

v-miRNA	Tissues	Target	Sites	Target's GO terms
miR-BART3-3p	BCLL, BL, HG, L, LBCL, MCL	CASP10	1	Pro-Apoptosis
miR-BART6-3p	BCLL, BL, HG, L, LBCL, MCL, NC	BAD	1	Pro-Apoptosis
miR-BART7	AML, BCLL, BL, ESC, GC, HG, LBCL, MCL, USSC	RB1	1	Negative regulation of cell cycle
miR-BART9	BCLL, BL, HG, MCL	RAD1	2	Cell cycle checkpoint
		RB1	1	Negative regulation of cell cycle
miR-BART10	BCLL, BL, GC, HG, MCL	ZAK	1	Cell cycle arrest
miR-BART11-5p	BCLL, BL, HG, L, NC	CASP3	1	Pro-Apoptosis
miR-BART11-3p	BCLL, BL, HG, L, MCL, NC	TP53	1	Regulation of cell cycle
miR-BART12	BCLL, BL, GC, NC, PEL	TP53	1	Regulation of cell cycle
miR-BART13	BCLL, BL, HG, L, MCL	CASP3	1	Pro-Apoptosis
		APC	2	Negative Regulation of cyclin-dependent kinase activity
miR-BART15	BCLL, BL, HG, MCL	CASP3	1	Pro-Apoptosis
miR-BART16	BCLL, BL, HG, L, MCL	BID	1	Pro-Apoptosis
miR-BART19	BL, HG, MCL	APBB2	2	Cell cycle arrest
		ZAK	1	Cell cycle arrest
		STK4	2	Pro-Apoptosis
miR-BART20-3p	BCLL, BL, L	BAX	1	Pro-Apoptosis
miR-BHRF1-1	BCLL, BL, HG, L, MCL	CUL4	2	Cell cycle arrest
miR-BHRF1-2	AML, BCLL, BL, ESC, HG, L, LBCL, M, MCL, LAC, LU, USSC	RAD1	1	Cell cycle checkpoint
miR-BHRF1-2*	-	CCNG2	1	Cell cycle checkpoint
		GAS1	2	Cell cycle arrest
		RB1	2	Negative regulation of cell cycle
miR-BHRF1-3	BCLL, BL, HG, L, LBCL, MCL	BID	1	Pro-Apoptosis

Table 2. Predicted targets for KSHV v-miRNAs. For each miRNA, the list of tissues where it is expressed, the predicted targets, the number of site on each target's transcript and the GO term associated to the targets are shown. Tissues legend: BCBL: Body cavity-based lymphoma, BL: Burkitt's lymphoma, PEL: Primary-effusion lymphoma.

v-miRNA	Tissues	Target	Sites	Target's GO terms
miR-K12-1	BCBL, BL, PEL	RBL1	1	Negative regulation of cell cycle
		RAD1	1	Cell cycle checkpoint
miR-K12-2	BCBL, BL, PEL	CASP10	1	Pro-Apoptosis
		APC	2	Negative Regulation of cyclin-dependent kinase activity
		RAD1	1	Cell cycle checkpoint
miR-K12-3	BCBL, BL, PEL	APC	1	Negative Regulation of cyclin-dependent kinase activity
		STK4	3	Pro-Apoptosis
miR-K12-4-5p	BCBL, BL, PEL	RBL1	1	Negative regulation of cell cycle
		ZAK	1	Cell cycle arrest
miR-K12-6-3p	BCBL, BL, PEL	BID	1	Pro-Apoptosis
		BTG3	1	Negative Regulation of mitotic cell cycle
miR-K12-9*	BCBL, BL, PEL	CASP8	1	Pro-Apoptosis
		TP53	1	Regulation of cell cycle
miR-K12-10a	BCBL, BL, PEL	CASP10	1	Pro-Apoptosis
miR-K12-11	BL	RB1	1	Negative regulation of cell cycle
		RBL1	1	Negative regulation of cell cycle
		APC	3	Negative Regulation of cyclin-dependent kinase activity
miR-K12-12	PEL	CASP10	1	Pro-Apoptosis

Table 3. Expression profiles of the predicted targets in infected cells. The profiles were retrieved from the NCBI GEO Profiles database. For each target, the GEO record ID, the virus, the mean value in the control sample (not infected), the mean value in the infected cell and the infected/control ratio are shown. Only values less than 1 are reported, since they indicate that the gene is under-expressed in the infected sample, compared to the control cell.

Gene	GEO Record	Virus	Mean (Control)	Mean (Infected)	Ratio (I/C)
ABL1	GDS989 - 1636_g_at	EBV	5928.75	3531.80	0.59
	GDS989 - 39730_at	EBV	5839.95	3504.20	0.60
	GDS989 - 1635_at	EBV	2786.60	1428.30	0.51
	GDS940 - 202123_s_at	KSHV	406.35	246.35	0.60
APC	GDS940 - 203525_s_at	KSHV	110.60	63.90	0.57
BID	GDS940 - 204493_at	KSHV	263.25	140.40	0.53
BTG3	GDS940 - 213134_x_at	KSHV	471.05	233.25	0.49
CCNG2	GDS989 - 1913_at	EBV	2690.40	1679.80	0.62
	GDS940 - 211559_s_at	KSHV	109.65	98.55	0.89
RAD1	GDS989 - 36857_at	EBV	2365.45	1954.6	0.82
	GDS940 - 204461_x_at	KSHV	176.50	77.55	0.43
	GDS940 - 204460_s_at	KSHV	136.75	73.85	0.54
	GDS940 - 210216_x_at	KSHV	158.55	102.85	0.64
RB1	GDS989 - 2044_s_at	EBV	1307.35	1149.60	0.87
	GDS940 - 203132_at	KSHV	157.80	140.90	0.89
SMAD3	GDS940 - 205398_s_at	KSHV	314.75	157.80	0.50
STK4	GDS940 - 211085_s_at	KSHV	19.65	12.10	0.61
TP53	GDS989 - 1974_s_at	EBV	1712.40	1561.70	0.91
	GDS989 - 1974_s_at	KSHV	1712.40	1530.25	0.89
	GDS940 - 201746_s_at	KSHV	405.70	370.00	0.91
ZAK	GDS940 - 218833_at	KSHV	42.50	30.25	0.71

prediction: RNA Hybrid, miRanda, RNA22 and PITA (Rehmsmeier et al, 2004; John et al, 2005; Huynh et al, 2006). For this purpose, we chose a set of validated v-miRNA/human targets for which binding sites details were available. Results are reported on Table 4. They show the capability of miRiam to identify all the validated binding

sites, in most cases as top ranking. We evaluated the statistical significance of our results by the Friedman rank test. While miRiam is comparable to PITA, in terms of number of correct sites identified, it outperforms the other tools in terms of the number of correct binding sites and their ranking.

Table 4. Comparison of miRiam to other target prediction tools. We chose a set of validated v-miRNA/human targets for which binding sites details were available. For each tested tool, the ranking of the site in the output is reported. A “-” symbol indicates that the tool wasn’t able to identify the site. P-values are computed by using the Friedman Rank Test, to assess whether or not miRiam significantly tends to perform better than the other tools across experimentally validated targets. Results show that miRiam performs better than RNA Hybrid, miRanda and RNA22, while is comparable to PITA.

Virus	miRNA	Target	Transcript	Site	miRiam	RNAHybrid	miRanda	RNA22	PITA
KSHV	miR-K12-11	BACH-1	NM_001186.2	3156-3162	2	2	-	-	3
KSHV	miR-K12-11	BACH-1	NM_001186.2	4504-4511	3	-	-	-	2
KSHV	miR-K12-11	BACH-1	NM_001186.2	4565-4571	1	-	-	-	1
KSHV	miR-K12-11	BACH-1	NM_001186.2	4714-4720	4	-	-	-	7
KSHV	miR-K12-11	BACH-1	NM_206866.1	3284-3290	2	3	-	-	3
KSHV	miR-K12-11	BACH-1	NM_206866.1	4632-4639	3	-	-	-	2
KSHV	miR-K12-11	BACH-1	NM_206866.1	4693-4699	1	-	-	-	1
KSHV	miR-K12-11	BACH-1	NM_206866.1	4842-4848	4	-	-	-	7
HCMV	miR-UL112-1	MICB	NM_005931.3	1319-1323	16*	1	2	-	-
EBV	miR-BART5	BBC3	NM_001127240.1	1366-1372	1	1	1	-	1
EBV	miR-BART5	BBC3	NM_001127241.1	1147-1153	1*	1	1	-	1
EBV	miR-BART5	BBC3	NM_001127242.1	956-962	1*	1	1	-	1
EBV	miR-BART5	BBC3	NM_014417.3	1450-1456	1	1	1	-	1
KSHV	miR-K5	BCLAF1	NM_014739.2	5301-5308	1	10	-	2	3
KSHV	miR-K5	BCLAF1	NM_001077440.1	5148-5155	1	9	-	2	3
KSHV	miR-K5	BCLAF1	NM_001077441.1	4782-4789	1	10	-	2	3
<i>p-values</i>					-	0.05	0.03	< 0.0001	0.25

*miRiam was able to identify these sites by relaxing the structural accessibility filter.

DISCUSSION

v-miRNAs vs human targets

Epstein Barr herpesvirus (EBV) is associated with several tumor types, such as Burkitt’s lymphoma, nasopharyngeal and gastric carcinoma (Damania, 2007). EBV expresses two families of miRNAs, which are located in the BART and BHRF1 genes (Pfeffer et al, 2004; Cai et al, 2006). The BHRF1 miRNAs are expressed in infected primary lymphoma, Burkitt’s lymphoma and lymphoblast cell lines during late latency and lytic stages, while BART members are always expressed in lymphoblasts in all latent stages. miR-BART12 has also been found in infected nasopharyngeal and gastric carcinoma cell lines (Kim et al, 2007).

Kaposi’s sarcoma-associated herpesvirus (KSHV) is associated with several forms of cancer like Kaposi’s sarcoma and primary effusion lymphomas (Damania, 2007). KSHV expresses an array of 13 miRNAs in infected primary effusion lymphoma and endothelial cell lines (Pfeffer et al, 2005; Marshall et al, 2007). miR-BHRF1-3, miR-BART16 in EBV and miR-K12-6-3p in KSHV show good matches to BID transcript. Moreover, BID mRNA levels decrease after viral infection. The BID silencing could cause the partial block of the connection between the extrinsic apoptotic pathway and the mitochondrial one. BID is a target of proteolytic activity of

CASP8, which is activated by cell surface death receptors. Truncated BID translocates in the mitochondria engaging BAX to trigger its pro-apoptotic activity, starting the release of the cytochrome c. This is a key event in the formation of the apoptosome complex (cytochrome c, APAF1 and CASP9) for the beginning of the intrinsic death pathway. Consequently, BID inhibition may lower cell sensitivity to external signals and promote resistance to apoptosis. Moreover, it has been shown that BHRF, the host gene of these miRNAs, encodes a protein which acts downstream of BID cleavage and upstream of mitochondrial damage, resulting in inhibition of TRAIL-induced apoptosis (Kawanishi et al, 2002). This makes plausible a function of these miRNAs as repressors of apoptosis.

TP53 is a predicted target for EBV miR-BART11-3p, miR-BART12 and KSHV miR-K12-9*. In particular two different favorable binding sites for both EBV-miR-BART12 and KSHV-miR-K12-9* have been identified in TP53 transcript. We also observed a significant decrease in TP53 transcript levels following EBV and KSHV infections (see Table 3). The presence of multiple binding sites for the same miRNA has been observed in several experimentally validated pairs. This potentially increases the degree of translational suppression. TP53 is an important regulator of cell cycle arrest, apoptosis, and cellular senescence and it may be activated by various forms of cellular stress, as viral

infection. Several reports provide evidence of how TP53 is involved in antiviral activity and how viruses in their infection strategy lead to protein inactivation of TP53 (Si and Robertson, 2006; Pampin et al. 2006). That being so, a further repression device of TP53 based on RNA Interference from viruses looks plausible. Moreover, some data suggest that functional TP53 can promote the lytic cycle of some viruses. Consequently a repression strategy controlled by viral microRNAs could be an efficient and inexpensive switch on / off system of TP53 activities.

Our data suggest that Cyclin G2 (CCNG2) could be target of miRNAs from both EBV and KSHV. This pro-apoptotic protein has been found to be downregulated in many different tumours (Choi et al, 2009), and a decrease in its activity has been associated with improved proliferation. Its key role in cell-cycle and cell plasticity in cancer is underlined by the observation that the therapeutic effect of rapamycin is also exerted through the enhancement of its transcription rate (Kasukabe et al, 2008).

The ability to interfere with tp53 pathway through the post transcriptional regulation of some of its fundamental components (tp53, BID, cyclin g2) may constitute a mechanism to tightly and efficiently control the host survival rate improving replication and diffusion of viral progeny. Similarly, other genes involved in apoptosis and in the regulation of cell cycle, BAD, BTG3, BAX, RAD1, ZAK and RB1, among others, are predicted to be targets of EBV and KSHV miRNAs. The repression of these genes may help the virus to gain control of the host cells, thus favoring the accomplishment of the viral life cycle.

v-miRNAs involvement in cancer

Viruses are often involved in cancer development. They may directly promote cell malignancy or facilitate the progression of already established tumors (Damania, 2007). While protein based mechanisms of interaction between virus and hosts have been widely described, a direct involvement of v-miRNAs in cancer is not yet demonstrated. However, since experimental evidences show the capability of v-miRNAs to perturb host genes expression (Barth et al, 2008; Klase et al, 2009), their contribution to tumor insurgence is plausible. Moreover, a miRNA based host regulation system may represent a faster way for viruses to counteract cellular response to infection promoting their latency. According to Pfeffer and Voinnet (Pfeffer and Voinnet, 2006), v-miRNAs could act as direct oncogenes or indirectly promote cancer development and diffusion, by supporting external etiologic agents.

The predictions presented in this study are consistent with these scenarios. In particular the inhibition of central nodes of the apoptotic pathways may lead to loss of cell cycle control by the cell itself.

CONCLUSIONS

The aim of this work was to produce a set of plausible hypotheses of involvement of v-miRNAs and human apoptosis genes in cancer development. In the study, we have presented computational predictions of v-miRNAs and human targets interactions, and show miRNA/target

duplexes which were energetically favourable and consistent with other experimentally validated pairs. Moreover, the target binding sites were characterized by high structural-accessibility for interactions with miRNAs. Future works will focus on providing experimental validations of such estimated interactions. Extension of predictions to other reasonable human transcripts, such as additional nodes of apoptosis pathway, cell-cycle regulators and genes involved in immune response, will be performed.

ACKNOWLEDGMENTS

The authors would like to thank Michael Levitt and Dennis Shasha for their helpful suggestions and comments, and the authors of the Vienna RNA Package for providing their software. This work was supported in part by Sicily Region Grants PROGETTO POR 3.14: “*Ricerca e Sviluppo suite di programmi per l’analisi biologica, denominata: BIOWARE*”.

COMPETING INTERESTS

None declared

LIST OF ABBREVIATIONS

miRNA: microRNA
v-miRNA: viral microRNA
EBV: Epstein-Barr herpesvirus
KSHV: Kaposi’s Sarcoma associated herpesvirus
SV40: Simian Virus 40
UTR: Un-translated region

REFERENCES

- Bartel DP. 2004. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*, 116, 281-297.
- Barth S, Pfuhl T, Mamiani A, et al. 2008. Epstein-Barr virus-encoded microRNA miR-BART2 down-regulates the viral DNA polymerase BALF5. *Nucleic Acids Res*, 36, 666-675.
- Cai X, Schäfer A, Lu S, et al. 2006. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathog*, 2, e23.
- Cai X and Cullen BR. 2006. Transcriptional origin of Kaposi’s sarcoma-associated herpesvirus microRNAs. *J Virol*, 80, 2234-2242.
- Choi MG, Noh JH, An JY, et al. 2009. Expression levels of cyclin G2, but not cyclin E, correlate with gastric cancer progression. *J Surg Res*, 157, 168-174.
- Choy EY, Siu KL, Kok KH, et al. 2008. An Epstein-Barr virus-encoded microRNA targets PUMA to promote host cell survival. *J Exp Med*, 205, 2551-2560.
- Cullen BR. 2006. Viruses and microRNAs. *Nature Genet*, 38 Suppl, S25-S30.
- Damania B. 2007. DNA tumor viruses and human cancer. *Trends Microbiol*, 15, 38-44.
- Garofalo M, Condorelli G and Croce CM. 2008. MicroRNAs in diseases and drug response. *Curr Opin Pharmacol*, 8, 661-667.
- Griffiths-Jones S, Saini HK, van Dongen S and Enright AJ. 2008. miRBase: tools for microRNA genomics. *Nucleic Acids Res*, 36 (Database Issue), D154-D158.
- Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP and Bartel DP. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*, 27, 91-105.
- Hausser J, Berninger P, Rodak C, Jantscher Y, Wirth S and Zavolan M. 2009. MirZ: an integrated microRNA expression

- atlas and target prediction resource. *Nucleic Acids Res*, 37 (Web Server Issue), W266-W272.
- Hofacker IL, Fontana W, Stadler PF, Bonhoeffer S, Tacker M and Schuster P. 1994. Fast Folding and Comparison of RNA Secondary Structures. *Monatshefte Chemie*, 125, 167-188.
- Huynh T, Miranda K, Tay Y, et al. A pattern-based method for the identification of microRNA-target sites and their corresponding RNA/RNA complexes. *Cell*, 126, 1203-1217.
- John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS. 2005. Human MicroRNA targets. *PLoS Biol*, 3, e264.
- Kasukabe T, Okabe-Kado J and Honma Y. 2008. Cotylenin A, a new differentiation inducer, and rapamycin cooperatively inhibit growth of cancer cells through induction of cyclin G2. *Cancer Sci*, 99, 1693-1698.
- Kawanishi M, Tada-Oikawa S and Kawanishi S. 2002. Epstein-Barr virus BHRF1 functions downstream of Bid cleavage and upstream of mitochondrial dysfunction to inhibit TRAIL-induced apoptosis in BJAB cells. *Biochem Biophys Res Commun*, 297, 682-687.
- Kertesz M, Iovino N, Unnerstall U, Gaul U and Segal E. 2007. The role of site accessibility in microRNA target recognition. *Nat Genet*, 39, 1278-1284.
- Kim do N, Chae HS, Oh ST, et al. 2007. Expression of viral microRNAs in Epstein-Barr virus-associated gastric carcinoma. *J Virol*, 81, 1033-1036.
- Klase Z, Winograd R, Davis J, et al. 2009. HIV-1 TAR miRNA protects against apoptosis by altering cellular gene expression. *Retrovirology*, 6, 18.
- Marshall V, Parks T, Bagni R, et al. 2007. Conservation of virally encoded microRNAs in Kaposi sarcoma-associated herpesvirus in primary effusion lymphoma cell lines and in patients with Kaposi sarcoma or multicentric Castleman disease. *J Infect Dis*, 195, 645-659.
- Mendez E, Salas-Ocampo E and Arias CF. 2004. Caspases mediate processing of the capsid precursor and cell release of human astroviruses. *J Virol*, 78, 8601-8608.
- Nair V and Zavolan M. 2006. Virus-encoded microRNAs: novel regulators of gene expression. *Trends Microbiol*, 14, 169-175.
- Pampin M, Simonin Y, Blondel B, Percherancier Y and Chelbi-Alix MK. 2006. Cross talk between PML and p53 during poliovirus infection: implications for antiviral defense. *J Virol*, 80, 8582-8592.
- Pfeffer S, Zavolan M, Grasser FA, et al. 2004. Identification of virus-encoded microRNAs. *Science*, 304, 734-736.
- Pfeffer S, Sewer A, Lagos-Quintana M, et al. 2005. Identification of microRNAs of the herpesvirus family. *Nature Methods*, 2, 269-276.
- Pfeffer S and Voinnet O. 2006. Viruses, microRNAs and cancer. *Oncogene*, 25, 6211-6219.
- Rehmsmeier M, Peter Steffen, Matthias Höchsmann and Robert Giegerich. 2004. Fast and effective prediction of microRNA/target duplexes. *RNA*, 10, 1507-1517.
- Robins H, Li Y and Padgett RW. 2005. Incorporating structure to predict microRNA targets. *Proc Natl Acad Sci USA*, 102, 4006-4009.
- Si H and Robertson ES. 2006. Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen induces chromosomal instability through inhibition of p53 function. *J Virol*, 80, 697-709.
- Zhu JY, Pfuhl T, Motsch N, et al. 2009. Identification of Novel Epstein-Barr Virus MicroRNA Genes from Nasopharyngeal Carcinomas. *J Virol*, 83, 3333-3341.

Supplementary Table S1. Predicted targets for EBV v-miRNAs. For each miRNA, the list of tissues where it is expressed, the predicted targets, the number of site on each target's transcript and the GO term associated to the targets are shown. Tissues legend: AML: Acute myeloid leukemia, BCLL: B-Chronic lymphocytic leukemia, BL: Burkitt's lymphoma, ESC: Embryonic stem cells, GC: Gastric carcinoma, HD: Hodgkin's disease, L: Lymphoblasts, LAC: Lung's adenocarcinoma, LBCL: Large B-cell lymphoma, LU: Lung, MCL: Mantle cell lymphoma, M: Myeloblasts, NC: Nasopharyngeal carcinoma, PEL: Primary-effusion lymphoma, USSC: Unrestricted somatic stem cells.

v-miRNA	Tissues	Target	Sites	Target's GO terms
miR-BART1-5p	BCLL, BL, HG, MCL	APC	1	Negative Regulation of cyclin-dependent kinase activity
		CUL2	1	Cell cycle arrest
		TBRG1	1	Cell cycle arrest
miR-BART2	BCLL, BL, HG, MCL	CDKN2A	1	Negative Regulation of cyclin-dependent kinase activity
		CDKN2B	1	Cell cycle arrest
		CUL3	1	Pro-Apoptosis
miR-BART3-3p	BCLL, BL, HG, L, LBCL, MCL	CASP10	1	Pro-Apoptosis
miR-BART5	BCLL, BL, HG, MCL	AIFM2	1	Pro-Apoptosis
		DAPK1	1	Pro-Apoptosis
miR-BART6-5p	BCLL, BL, HG, LBCL, MCL	GAS7	1	Cell cycle arrest
miR-BART6-3p	BCLL, BL, HG, L, LBCL, MCL, NC	BAD	1	Pro-Apoptosis
miR-BART7	AML, BCLL, BL, ESC, GC, HG, LBCL, MCL, USSC	CASP6	1	Pro-Apoptosis
		CUL3	1	Pro-Apoptosis
		EIF4G2	1	Cell cycle arrest
		FOXO4	1	Cell cycle arrest
		RB1	1	Negative regulation of cell cycle
miR-BART8-3p	AML, BCLL, BL, HG, LBCL, MCL	APBB2	1	Cell cycle arrest
		EIF4G2	1	Cell cycle arrest
		FEM1B	1	Pro-Apoptosis
		GAS1	1	Cell cycle arrest
		SIPA1	1	Negative regulation of cell cycle
		TP53BP2	1	Negative regulation of cell cycle
miR-BART9	BCLL, BL, HG, MCL	RAD1	2	Cell cycle checkpoint
		RB1	1	Negative regulation of cell cycle
		APBB2	1	Cell cycle arrest
		STK3	1	Pro-Apoptosis
miR-BART10	BCLL, BL, GC, HG, MCL	CDKN2C	1	Cell cycle arrest
		CUL5	1	Cell cycle arrest
		ZAK	1	Cell cycle arrest
miR-BART11-5p	BCLL, BL, HG, L, NC	CASP3	1	Pro-Apoptosis
		CUL2	1	Cell cycle arrest
miR-BART11-3p	BCLL, BL, HG, L, MCL, NC	TP53	1	Regulation of cell cycle
miR-BART12	BCLL, BL, GC, NC, PEL	TP53	1	Regulation of cell cycle
		APBB2	1	Cell cycle arrest
miR-BART13	BCLL, BL, HG, L, MCL	APC	2	Negative Regulation of cyclin-dependent kinase activity
		CASP3	1	Pro-Apoptosis
		CDKN2B	1	Cell cycle arrest
		MAPK1	1	Pro-Apoptosis
miR-BART14-3p	BCLL, BL, HG, MCL	ABL1	1	Pro-Apoptosis
		BCL211	1	Pro-Apoptosis
		CDKN2D	1	Cell cycle arrest
		DIABLO1	1	Pro-Apoptosis
		PAWR	1	Pro-Apoptosis
		STK4	1	Pro-Apoptosis
miR-BART15	BCLL, BL, HG, MCL	CASP3	1	Pro-Apoptosis
		CUL3	1	Pro-Apoptosis
		CUL5	1	Cell cycle arrest
		DAPK1	1	Pro-Apoptosis
miR-BART16	BCLL, BL, HG, L, MCL	BID	1	Pro-Apoptosis
		CIDEB	1	Pro-Apoptosis

		DHCR24	1	Cell cycle arrest
miR-BART17-3p	BCLL, BL, MCL	BOK	1	Pro-Apoptosis
		TRAF3	1	Pro-Apoptosis
miR-BART18	BCLL, BL, HG, MCL	APC	1	Negative Regulation of cyclin-dependent kinase activity
		LTA	1	Pro-Apoptosis
miR-BART19	BL, HG, MCL	APBB2	2	Cell cycle arrest
		APC	1	Negative Regulation of cyclin-dependent kinase activity
		BTG4	1	Cell cycle arrest
		CDKN2C	1	Cell cycle arrest
		IFNW1	1	Cell cycle arrest
		MAPK1	1	Pro-Apoptosis
		MPHOSPH1	1	Cell cycle arrest
		NLRP1	1	Pro-Apoptosis
		STK4	2	Pro-Apoptosis
		TNFRSF25	1	Pro-Apoptosis
		ZAK	1	Cell cycle arrest
miR-BART20-3p	BCLL, BL, L	APC	1	Negative Regulation of cyclin-dependent kinase activity
		BAX	1	Pro-Apoptosis
		CDKN1B	1	Pro-Apoptosis
		DHCR24	1	Cell cycle arrest
		ERN1	1	Cell cycle arrest
		NDUFA13	1	Pro-Apoptosis
		RUNX3	1	Negative regulation of cell cycle
miR-BHRF1-1	BCLL, BL, HG, L, MCL	BCL211	1	Pro-Apoptosis
		BIK	1	Pro-Apoptosis
		CUL4A	2	Cell cycle arrest
		EI24	1	Pro-Apoptosis
		LATS2	1	Negative Regulation of cyclin-dependent kinase activity
		MAPK1	2	Pro-Apoptosis
		TRAF3	1	Pro-Apoptosis
miR-BHRF1-2	AML, BCLL, BL, ESC, HG, L, LBCL, M, MCL, LAC, LU, USSC	CUL1	1	Cell cycle arrest
		RAD1	1	Cell cycle checkpoint
miR-BHRF1-2*	-	APC	1	Negative Regulation of cyclin-dependent kinase activity
		BNIP3	1	Pro-Apoptosis
		CCNG2	1	Cell cycle checkpoint
		CUL1	1	Cell cycle arrest
		CUL3	1	Pro-Apoptosis
		DAXX	1	Pro-Apoptosis
		DEDD	1	Pro-Apoptosis
		DIABLO	1	Pro-Apoptosis
		ERN1	1	Cell cycle arrest
		FOXO3A	1	Pro-Apoptosis
		GAS1	2	Cell cycle arrest
		ING4	1	Cell cycle arrest
		RB1	2	Negative regulation of cell cycle
		STK4	1	Pro-Apoptosis
TSPYL2	1	Negative regulation of cell cycle		
miR-BHRF1-3	BCLL, BL, HG, L, LBCL, MCL	BID	1	Pro-Apoptosis

Supplementary Table S2. Predicted targets for KSHV v-miRNAs. For each miRNA, the list of tissues where it is expressed, the predicted targets, the number of site on each target's transcript and the GO term associated to the targets are shown. Tissues legend: BCBL: Body cavity-based lymphoma, BL: Burkitt's lymphoma, PEL: Primary-effusion lymphoma.

v-miRNA	Tissues	Target	Sites	Target's GO terms
miR-K12-1	BCBL, BL, PEL	FOXO3A	1	Pro-Apoptosis
		HBP1	1	Cell cycle arrest
		PDCD4	1	Negative regulation of cell cycle
		PCDC6	1	Negative regulation of cell cycle
		RBL1	1	Negative regulation of cell cycle
		RAD1	1	Cell cycle checkpoint
miR-K12-2	BCBL, BL, PEL	TNFRSF10A	2	Pro-Apoptosis
		APC	2	Negative Regulation of cyclin-dependent kinase activity
		CASP10a	1	Pro-Apoptosis
		CDKN2C	1	Cell cycle arrest
		CUL1	1	Cell cycle arrest
		CUL3	1	Pro-Apoptosis
		CUL5	1	Cell cycle arrest
		LATS2	1	Negative Regulation of cyclin-dependent kinase activity
		MAPK1	1	Pro-Apoptosis
		RAD1	1	Cell cycle checkpoint
miR-K12-3	BCBL, BL, PEL	RBBP8	1	Cell cycle checkpoint
		STK4	1	Pro-Apoptosis
		APBB1	1	Cell cycle arrest
		APC	1	Negative Regulation of cyclin-dependent kinase activity
		CDKN2B	1	Cell cycle arrest
		CUL5	2	Cell cycle arrest
		FAF1	1	Pro-Apoptosis
		RASSF1	1	Cell cycle arrest
		RBBP8	2	Cell cycle checkpoint
		SMAD3	2	Cell cycle arrest
miR-K12-4-5p	BCBL, BL, PEL	STK3	1	Pro-Apoptosis
		STK4	3	Pro-Apoptosis
		CCNG2	1	Cell cycle checkpoint
		ING1	1	Negative regulation of cell cycle
miR-K12-6-3p	BCBL, BL, PEL	RBL1	1	Negative regulation of cell cycle
		ZAK	1	Cell cycle arrest
		BID	1	Pro-Apoptosis
miR-K12-7	PEL	BTG3	1	Negative Regulation of mitotic cell cycle
		TBRG1	1	Cell cycle arrest
		ABL1	1	Pro-Apoptosis
miR-K12-9	PEL	FOXO4	1	Cell cycle arrest
		TBRG1	1	Cell cycle arrest
miR-K12-9*	BCBL, BL, PEL	CASP8a	1	Pro-Apoptosis
		TP53	1	Regulation of cell cycle
miR-K12-10a	BCBL, BL, PEL	CASP10a	1	Pro-Apoptosis
		CASP10d	1	Pro-Apoptosis
		CUL2	2	Cell cycle arrest
		CUL4a	1	Cell cycle arrest
		EI24	1	Pro-Apoptosis
		FOXO4	1	Cell cycle arrest
miR-K12-10b	PEL	SMAD3	1	Cell cycle arrest
		GAS7	1	Cell cycle arrest
miR-K12-11	BL	BCL10	1	Pro-Apoptosis
		RB1	1	Negative regulation of cell cycle
		RBL1	1	Negative regulation of cell cycle
		APC	3	Negative Regulation of cyclin-dependent kinase activity
miR-K12-12	PEL	MPHOSPH1	1	Cell cycle arrest
		CASP10a	1	Pro-Apoptosis

Supplementary Table S3. Expression profiles of the predicted targets in infected cells. The profiles were retrieved from the NCBI GEO Profiles database. For each target, the GEO record ID, the virus, the mean value in the control sample (not infected), the mean value in the infected cell and the infected/control ratio are shown. Only values less than 1 are reported, since they indicate that the gene is under-expressed in the infected sample, compared to the control cell.

Gene	GEO Record	Virus	Mean (Control)	Mean (Infected)	Ratio (I/C)
ABL1	GDS989 - 1636_g_at	EBV	5928.75	3531.80	0.59
	GDS989 - 39730_at	EBV	5839.95	3504.20	0.60
	GDS989 - 1635_at	EBV	2786.60	1428.30	0.51
	GDS940 - 202123_s_at	KSHV	406.35	246.35	0.60
APC	GDS940 - 203525_s_at	KSHV	110.60	63.90	0.57
BCL10	GDS940 - 205263_at	KSHV	637.50	439.55	0.68
BID	GDS940 - 204493_at	KSHV	263.25	140.40	0.53
BTG3	GDS940 - 213134_x_at	KSHV	471.05	233.25	0.49
CCNG2	GDS989 - 1913_at	EBV	2690.40	1679.80	0.62
	GDS940 - 211559_s_at	KSHV	109.65	98.55	0.89
CDKN2C	GDS940 - 204159_at	KSHV	116.10	68.95	0.59
CUL1	GDS940 - 207614_s_at	KSHV	266.50	214.25	0.80
CUL2	GDS989 - 37894_at	EBV	817.95	454.50	0.55
	GDS940 - 203079_s_at	KSHV	254.00	164.40	0.64
CUL3	GDS940 - 201371_s_at	KSHV	766.05	533.75	0.69
CUL4A	GDS940 - 201423_s_at	KSHV	270.50	208.75	0.77
CUL5	GDS940 - 203531_at	KSHV	323.30	256.55	0.79
EI24	GDS940 - 216396_s_at	KSHV	349.60	202.05	0.57
FAF1	GDS940 - 218080_x_at	KSHV	205.95	123.65	0.60
FOXO3A	GDS940 - 204132_s_at /	KSHV	62.15	55.60	0.89
HBP1	GDS940 - 209102_s_at	KSHV	281.35	274.65	0.97
ING1	GDS940 - 209808_x_at	KSHV	73.90	61.05	0.82
MAPK1	GDS989 - 976_s_at	EBV	1619.10	898.90	0.55
	GDS940 - 212271_at	KSHV	750.10	399.50	0.53
MPHOSPH1	GDS940 - 205235_s_at	KSHV	120.15	54.95	0.45
RAD1	GDS989 - 36857_at	EBV	2365.45	1954.6	0.82
	GDS940 - 204461_x_at	KSHV	176.50	77.55	0.43
	GDS940 - 204460_s_at	KSHV	136.75	73.85	0.54
	GDS940 - 210216_x_at	KSHV	158.55	102.85	0.64
RASSF1	GDS940 - 204346_s_at	KSHV	116.65	73.90	0.63
RB1	GDS989 - 2044_s_at	EBV	1307.35	1149.60	0.87
	GDS940 - 203132_at	KSHV	157.80	140.90	0.89
	GDS940 - 211540_s_at	KSHV	50.60	44.70	0.88
RBBP8	GDS940 - 203344_s_at	KSHV	200.60	112.40	0.56
SMAD3	GDS940 - 205398_s_at	KSHV	314.75	157.80	0.50
STK3	GDS940 / 208855_s_at	KSHV	933.70	642.40	0.68
STK4	GDS940 - 211085_s_at	KSHV	19.65	12.10	0.61
TP53	GDS989 - 1974_s_at	EBV	1712.40	1561.70	0.91
	GDS989 - 1974_s_at	KSHV	1712.40	1530.25	0.89
	GDS940 - 201746_s_at	KSHV	405.70	370.00	0.91
ZAK	GDS940 - 218833_at	KSHV	42.50	30.25	0.71