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Exploring the major cross-talking edges of competitive endogenous RNA networks in human Chronic and Acute Myeloid Leukemia

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ABSTRACT

Background: Human Chronic and Acute Myeloid Leukemia are myeloproliferative disorders in myeloid lineage of blood cells characterized by accumulation of aberrant white blood cells. In cancer, the anomalous transcriptome includes deregulated expression of non-coding RNAs in conjunction with protein-coding mRNAs in human genome. The coding or non-coding RNA transcripts harboring miRNA-binding sites can converse with and regulate each other by explicitly contending for a limited pool of shared miRNAs and act as competitive endogenous RNAs (ceRNAs). An unifying hypothesis attributing 'modulation of expression of transcripts' in this fashion had been defined as 'competitive endogenous RNA hypothesis'. Network built with ceRNAs evidently offers a platform to elucidate complex regulatory interactions at post-transcriptional level in human cancers.
Methods: Contemplating cancers of human myeloid lineage we constructed ceRNA networks for CML and AML coding and non-coding repertoire utilizing patient sample data. Through functional enrichment analysis we selected the significant functional modules for transcripts being differentially expressed in Blastic phases of each cancer types with respect to Normal. After retrieving free energy of binding and duplex formation of shared miRNAs on ceRNAs, we performed statistical averaging of energy values over the ensemble of populations considering cellular system as in canonical (Iso-thermal) situation.
Results and conclusions: We aimed to shed light on 'Sibling Rivalry' in ceRNA partners from the perspective of statistical thermodynamics, identified major cross-talking tracks and ceRNAs influencing transcripts concerned in myeloid cancer systems.
General significance: Insights into ceRNA-regulation will shed light on progression and prognosis of human Chronic and Acute Myeloid Leukemia.

1. Introduction

MicroRNAs (miRNAs), a newly discovered class of regulators in the gene network, are small, evolutionarily conserved and single-stranded non-coding RNA molecules which are getting crucial importance for regulation at the posttranscriptional level of gene expression [1]. Mature miRNAs, which are generated through two-step cleavage of primary miRNA (pri-miRNA), are incorporated into the effector complex named RNA-induced silencing complex (RISC) and function as a guide by base-pairing with target mRNA to negatively regulate its expression [2]. They are seen to degrade or translationally inhibit the target mRNAs through binding with the miRNA response elements (MREs) on 3′ untranslated regions (UTRs) of target RNA transcripts with imperfect complementarity [3]. As the mature miRNAs are short oligonucleotides (typically 21 nucleotide long), each one may recognize many mRNAs. Every individual mRNA usually offers MREs for multiple miRNAs and simultaneously, an miRNA can bind to multiple target coding and non-coding gene products with various affinity. Protein coding mRNA transcripts with set of shared miRNAs, thus, can communicate with other coding or non-coding transcripts through a 'microRNA response

Abbreviations: AML, Acute Myeloid Leukemia; CML, Chronic Myeloid Leukemia; ceRNA, Competitive endogenous RNA; ceRNET, competitive endogenous RNA network; miRNA, microRNA; MRE, microRNA response elements

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element' based language which attributes the coding ones a novel non-coding function encrypted in the mRNA itself [4]. The cross-talking of multiple mRNAs to be targeted by shared miRNAs has been termed as ceRNA cross-regulation while the participating transcripts act as individual ceRNA molecules. The complex networks where miRNAs and their corresponding target mRNAs are connected with each other are termed as ceRNA networks or ceRNETs. It was hypothesized that the expression levels of the ceRNET components, influence the cross-regulation in a manner such that when the total number of transcripts vastly exceeds the number of miRNA molecules, the overall ceRNA activity becomes minimal due to the limited number of available miRNAs, and conversely, if the miRNA molecules become more abundant than the ceRNA molecules, cross-regulation is unlikely to occur as most transcripts are fully repressed [5, 6]. Thus, the optimal ceRNA-mediated cross-regulation takes place at a near-equimolar equilibrium of all network elements [5]. ceRNA regulation introduces a new layer of intricacy in miRNA-based cancer therapies which was reflected in a number of research articles on transcriptomics throughout the last decade. The pioneering work of Poliseno et al. demonstrated ceRNA cross regulation in the context of *PTEN* (phosphatase and tensin homolog, a key tumor suppressor) and its pseudogene *PTENP1* whose abundance determines critical outcomes in tumorigenesis [7]. *PTENP1*, also acknowledged as a bona fide tumor suppressor gene, was observed to retain the ability to regulate the cellular levels of *PTEN* by acting as a perfect 'miRNA decoy' or ceRNA for its parental gene. A similar but reverse trend was observed in *KRAS* and its pseudogene *KRAS1P* where the latter acts as a ceRNA of *KRAS* for the shared pool of miRNAs and their abundance are directly correlated in prostate cancer, neuroblastoma, retinoblastoma and hepatocellular carcinoma indicating a proto-oncogenic role of *KRAS1P* in cancer [3].

In this context of cancer research, it is worth notifying that, over the last 4 decades, investigation on human Myeloid Leukemia has fueled progress in a number of key areas [8, 9], particularly in the identification of leukemia-associated oncogenes and tumor suppressors [10]. Thus, a delineation of human myeloid malignancy from the perspective of ceRNA network becomes imperative owing to the dearth of study on regulatory networks in both Chronic and Acute Myeloid Leukemia. Chronic Myeloid Leukemia (CML) is a biphasic carcinoma of bone marrow that is identified commonly by the presence of abnormally short Philadelphia chromosome and sometimes by other genomic variations [11]. On the other hand, Acute Myeloid Leukemia (AML) is a highly heterogeneous disease categorized by large chromosomal translocations and subsequently mutations in the genes involved in hematopoietic proliferation and differentiation [12]. In a recent report, it was stated that protein-coding transcripts in conjunction with the non-coding ones can converse with each other via their shared miRNAs and the altered ceRNA landscape in cancer provides an indication of putative biomarkers and plausible drug targets in CML therapy [13]. To further illuminate the complexity of ceRNA regulation in human myeloid cancer, we here, retrieved significant functional modules from the ceRNA network (ceRNET) built on human CML and AML patient sample data. Through an approach of statistical thermodynamics we probed into the intricacy of ceRNA rivalry in those selected modules. Thermodynamic favorability and site accessibility of miRNA-mRNA duplex were analyzed by secondary structure of the duplex and free energy calculations [14] in the formation of Watson-Crick base pairs within miRNA:mRNA constructs [15, 16]. Thereafter, in the selected functional modules within Leukemia ceRNETs, we showed that when $miRNA_x$ has an ability to bind to $mRNA_a$ as well as $mRNA_b$ with equal free energy then the two transcripts go for a competition to take over the possession of the same miRNA molecule, can form a miRNA-target mRNA network and act as ceRNA for each other. Such equal free energy is, however, mostly not mandatory, and is not likely to happen also. In that scenario, the rivalry turns out to be an unfair one when the difference in binding energy becomes huge and the mRNA offering more negative free energy for duplex formation sponges the shared miRNAs allowing its competitor to be expressed. In a nutshell, the significant cross-talking neighbors with substantial implication in myeloid leukemia pathogenesis were identified in our study by dint of statistical thermodynamics and computational approach. Further Q-PCR validation was carried out for the selected functional modules in CML and AML systems respectively.

2. Material and methods

2.1. Retrieval of CML and AML data for coding and non-coding transcripts

Patient sample data corresponding to mRNA expression in CML, AML and healthy individuals were obtained from GEO (Gene Expression Omnibus) (http://www.ncbi.nlm.nih.gov/geo/) (Accession No. GSE47927, GSE48558), Pan-ceRNADB [17], starBase v2.0 [18] and DisGeNet [19]. miRNA expression data was attained from PhenomiR 2.0 [20]; HMDD v2.0 (the human microRNA disease database) (http://202.38.126.151/hmdd/mirna/md/) [21]; miRCancer–microRNA Cancer Association Database [22], ENCODE [23] and GENCODE (http://www.gencodegenes.org) [24] (Supplementary Table S1).

2.2. Identification of miRNA target genes

miRNA target gene information was obtained from microRNA.org [25], TargetScan v7 [26], miRTarBase [27], EMBL-EBI MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), PicTar web interface [28], and lnCeDB (Human) [29].

2.3. Selection of candidate ceRNA pairs

Putative ceRNA pairs were formed from the intersection set of expressed transcripts in Leukemia system and miRNA target transcript pool using in-house Perl program. The probability of the transcripts in individual pairs, of being ceRNA of one another, was assessed using a Hypergeometric cumulative distribution function test [30] considering the number of shared miRNAs between the ceRNA pair against the total number of miRNAs targeting each transcript in that particular pair. A *p*-value was calculated for each pair using the formula:

$$p = \sum_{i=c}^{\min(K,n)} \frac{\binom{K}{i}\binom{N-K}{n-i}}{\binom{N}{n}}$$

where, N = Total number of miRNAs in that particular cell line,n, K = Number of miRNAs interacting with the transcripts (mRNA or lncRNA or pseudogene), and c = Number of miRNAs shared between the ceRNA pair.

The ceRNA pairs with a *p*-value $< 10^{-5}$ were considered as probable ceRNA pairs.

2.4. Identification of the co-ordinates of the miRNA response elements (MREs)

Co-ordinates of miRNA Response Elements in miRNA target transcript set were identified using in-house Perl program considering all possible miRNA seed sites: 6mer, 7mer-m8, 7mer-A1, 8mer, Offset 6mer and 3′ compensatory site. [31, 32] MRE locations were further verified using FINDTAR3 – a miRNA target prediction and analysis tool (http://bio.sz.tsinghua.edu.cn) [33]. Transcript sequences were obtained from UCSC Genome Browser (https://genome.ucsc.edu/) [34]. Sequences of mature miRNAs were attained from miRBase (http://www.mirbase.org/) [35].

2.5. Calculation of ceRNA score

A ceRNA score for each probable ceRNA pair was assigned by taking into consideration the number of shared MREs between the pair [29].

$$\text{ceRNA score of a certain transcript within a pair} = \frac{\text{the number of MREs for the distinct shared miRNAs between the pair}}{\text{the total number of MREs for all distinct miRNAs targeting that particular ceRNA}}$$

2.6. Construction of CML, AML and normal ceRNA network

The CML, AML and Normal ceRNA network (ceRNET) were constructed considering the interaction between all selected ceRNA pairs and visualized using Cytoscape v3.5.1. [36].

2.7. Functional annotation of CML and AML-specific ceRNET

The functional enrichment analysis of all interacting pairs in CML and AML ceRNET was performed using DAVID [37] considering data from OMIM Disease, COG Ontology, GO Term - Biological processes, Cellular component, Molecular function and KEGG Pathway. All *p*-values were subject to false discovery rate (Benjamini Hochberg FDR) correction.

2.8. Estimation of free energy of binding and duplex formation between miRNA and ceRNAs

Information regarding free energy of binding and duplex formation between the shared miRNAs and the ceRNA target transcripts was obtained from RNAfold web server of ViennaRNA web services [38]. The RNAup program [39], embedded in that package, models the entire binding energy for the interaction at a particular site as $\Delta G_{binding} = \Delta G_u{}^A + \Delta G_u{}^B + \Delta G_h$ where $\Delta G_u{}^{A,B}$ are the free energy required to make the binding region in molecules A or B (coding/non-coding RNA and miRNA in our case) accessible by removing intra-molecular structure and ΔG_h denotes the free energy gained from forming the inter-molecular duplex. RNAup hence firstly evaluates the accessibility or the energy necessary to open-up the structure for every stretch of bases up to a certain length for both RNA molecules. Subsequently, the interaction free energy is computed and combined with the opening energy for the entire micro-states (thermodynamically termed as ensemble) to get the total binding energy [40].

2.9. Statistical averaging of the binding energy for all micro-states

The ensemble average of free energy values for all micro-sates formed in different MREs present on a transcript for a certain miRNA is represented as

$$<Q> = \frac{\sum_i q_i e^{-G_i/k_B T}}{\sum_i e^{-G_i/k_B T}}$$

where q_i is free energy, k_B is Boltzmann constant, T is absolute temperature of the system (300 K as physiological temperature) and G_i values are obtained from RNAfold as discussed above [41]. The denominator or the Partition Function shows the contribution from all transcripts in a ceRNA set harboring MREs for that miRNA as the miRNA concerned can target other ceRNAs present in a particular ceRNA sub-network.

2.10. Co-expression analysis in CML and AML sub-ceRNETs

2.10.1. Cell culture

K562 cells (CML cell line) were procured from National Centre for Cell Science, Pune, India (http://www.nccs.res.in/). The cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Invitrogen, Pittsburgh, PA). THP1 cells (AML cell line) [42] were kindly donated by Professor Subrata Banerjee (Biophysics and Structural Genomics Division, Saha Institute of Nuclear Physics, Kolkata) and were maintained in RPMI-1640 (Gibco). Both cell lines were grown in respective medium supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin-streptomycin (10 µl/ml of medium, Gibco) at 37 °C incubator with 5% (*v*/v) CO_2. Both the cells, when reached the density of 7×10^6 cells/ml in T25 flask, were given split by centrifugation at 1000 rpm for 5 min at room temperature (RT) followed by seeding in respective culture plate as per the experimental necessity.

2.10.2. RNA isolation

Cells were seeded in 35 mm culture dish and when reached the confluency of 1.5–2 × 10^6 cells/ml were used for RNA extraction. As both the cells grow in suspension, they were taken in 2 ml micro centrifuge tubes and after spinning at 1000 rpm for 5 min. at RT, the supernatant was discarded and the cell pellet was resuspended in 1 ml TRI-reagent (Sigma) for isolation of total RNA following the standard protocol (Sigma).

2.10.3. Quantitative polymerase chain reaction (Q-PCR)

1 µg of total RNA was first reverse transcribed into cDNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific) as manufacturer's instruction and subsequently was used as template in Q-PCR assay for relative quantification of specific transcript, performed on the ABI 7500 Real Time PCR system (Applied Biosystems). 2× Power SYBR GREEN mix (Applied Biosystems) was used in this reaction along with specific primers designed by PrimerQuest tool of Integrated DNA technologies (IDT). The amplification cycle (total 40 cycles) was set as: 95 °C for 30 s, Tm for 30 s, 72 °C for 30 s. The primer sequences are mentioned in Supplementary Table S2.

3. Results

3.1. Construction of CML and AML specific ceRNA network

We obtained a total number of 22,126 and 24,521 transcripts in CML and AML Blastic phases of patient sample data [43]. We then retrieved 7158 and 3555 miRNA-mediated ceRNA cross-talking sub-network (each sub-network contains a coding transcript with its significant ceRNA pairs) from the gene expression data, miRNA expression and miRNA target gene data of CML and AML respectively. From these two sets of data, we constructed CML (number of nodes: 7188) and AML (number of nodes: 4525) ceRNA networks (ceRNETs) based on shared miRNAs and MREs.

3.2. Selection of the significant ceRNA sets in CML and AML ceRNETs

From all significant ceRNA interacting pairs we chose only the transcripts those are being significantly and differentially expressed in Blastic phase with respect to Normal in the gene expression data of two myeloid cancers. Then, we performed Functional Enrichment analysis for all the ceRNETs comprising those transcripts. We selected the ceRNA sub-ceRNETs (Table 1) specifically showing a functional enrichment in the terms 'Chronic Myeloid Leukemia' and 'Acute Myeloid Leukemia' (with *p*-value < .05 and fold enrichment value > 10).

Accordingly, we obtained 6 (*CDKN1A*, *ABL1*, *BTN2A1*, *ENPP1*, *CNST* and *SYNM*) and 2 (*CLOCK* and *SUZ12*) ceRNA sub-ceRNETs for CML and AML respectively. From herein the transcripts associated with the above mentioned sub-ceRNETs would be referred to as 'Transcripts of Interest'. Supplementary Fig. S3 demonstrates co-expressive nature of the 'Transcripts of Interest' with corresponding ceRNAs in CML and AML cell lines which support the previous observation that transcripts sharing the most miRNA binding sites are co-expressed (4). Sub-

Table 1
Functional Enrichment Analysis result of the selected transcripts with their ceRNAs enriched in the terms 'Chronic' and 'Acute Myelogenous Leukemia'.

Enrichment GO term	Transcripts of interest	*p*-Value	Benjamini Hochberg FDR
Chronic myeloid leukemia	CDKN1A	3.4e−2	7.2e−1
	ABL1	4.8e−2	7.2e−1
	BTN2A1	4.5e−2	6.5e−1
	CNST	2.8e−2	4.7e−1
	ENPP1	4.5e−2	4.5e−1
	SYNM	1.2e−3	1.4e−1
Acute myeloid leukemia	CLOCK	4.7e−2	4.9e−1
	SUZ12	2.5e−2	5.5e−1

ceRNETs for *CDKN1A* (CML system) and *CLOCK* (AML system) were provided as examples in Fig. 1 (A, B).

3.3. Competitive binding of miRNAs on different MREs on a ceRNA

To delve deeper in the intricacy of miRNA sharing among ceRNAs, we assessed the miRNA binding probabilities and consequently miRNA sponging effect. We estimated the free-energy of each individual double helix formation by a short miRNA with its complementary region of mRNA by adding free-energies of duplex formation by the constituent dinucleotide steps. The simple averaging of the free energy values is, however, not much meaningful when multiple competitive binding sites are available for the miRNA in a particular transcript or even in other ceRNAs in a sub-ceRNET and hence the concept of statistical mechanical weighted averaging was needed in our analysis.

As an miRNA can have multiple binding sites on an mRNA with different levels of complementarity (length as well as sequence), giving

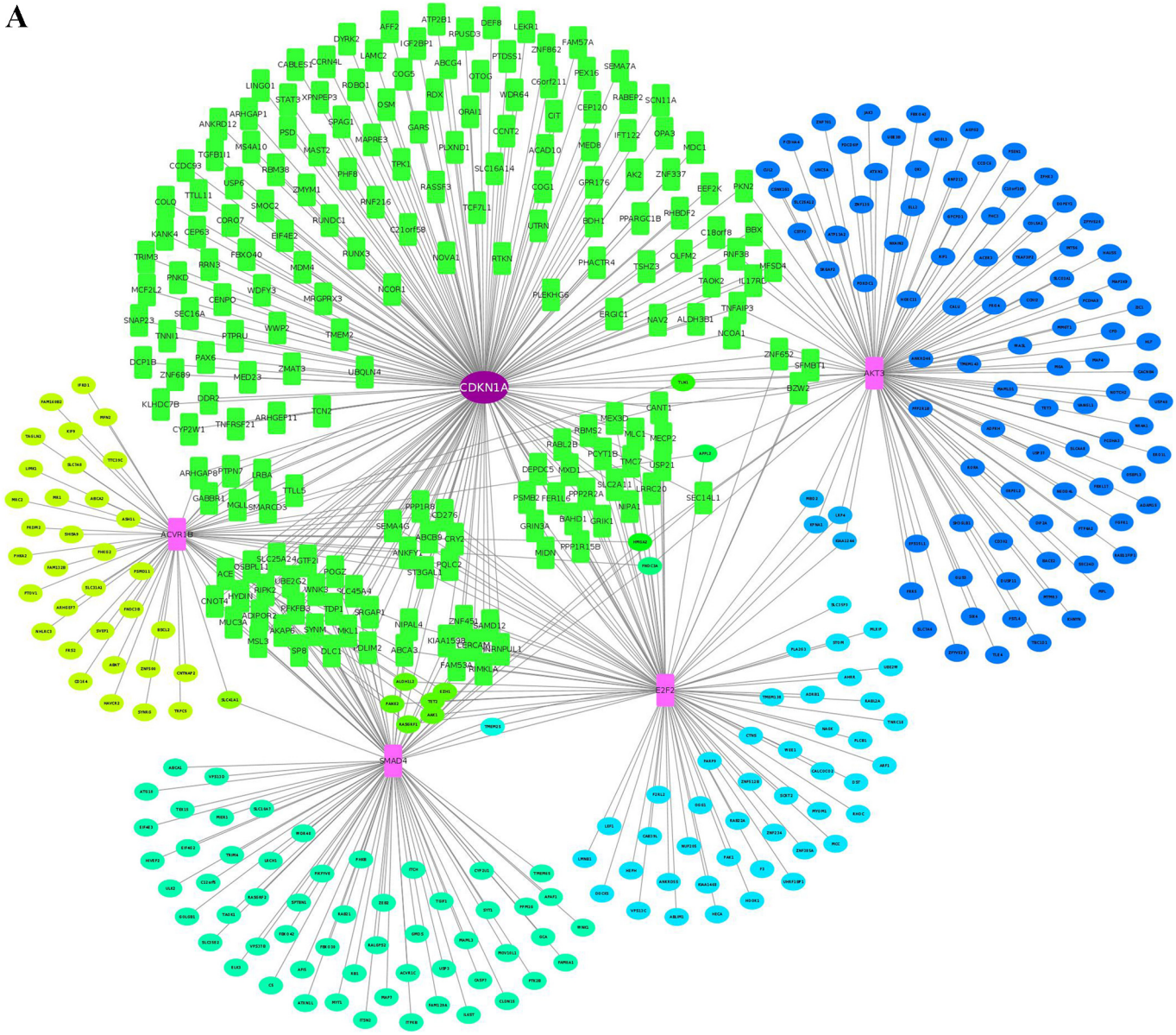

Fig. 1. (A, B): Sub-ceRNETs for *CDKN1A* (CML system) and *CLOCK* (AML system). 'Transcripts of Interest' *CDKN1A* (A) and *CLOCK* (B) are labeled in magenta round shaped icon; Associated ceRNAs in green round rectangle; Other nodes are in oval shaped icon and the colors are fixed by applying Neighborhood Connectivity and Discrete mapping value generators (Rainbow) in Cytoscape (v3.5.1) node style. ceRNAs with significant cross-talking in Leukemia are labeled in pink round rectangle icon.

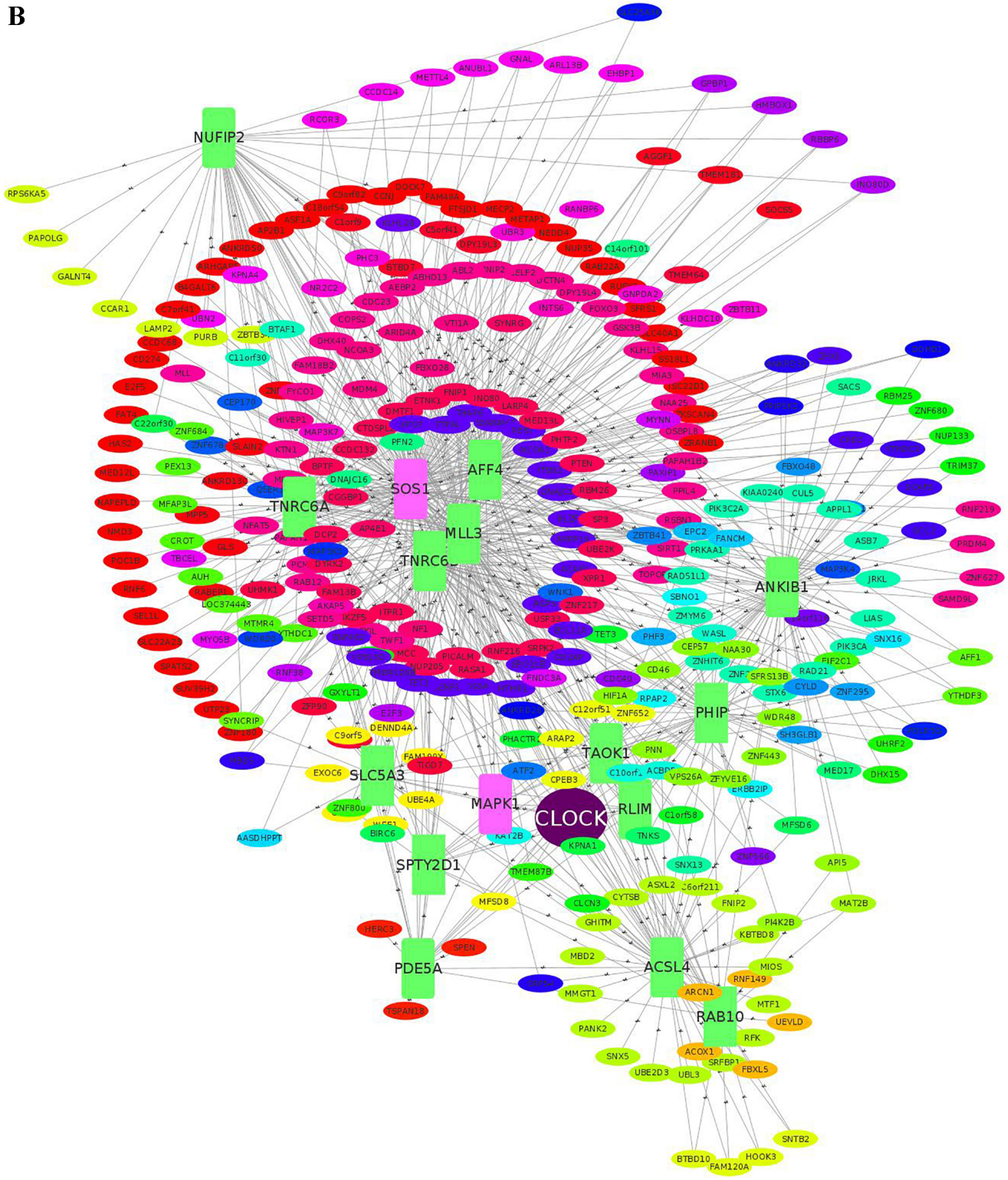

Fig. 1. (*continued*)

rise to nearly equal or even different free-energy values, again a competition arises as on which site the miRNA would prefer to bind where the preference of binding to a particular site is expected to be determined by the free-energy value. The miRNA further can, in principle, hop from one site of an mRNA to another. The time spent by an miRNA on a given site of an mRNA would depend on the free energy arising from complementary sequence [44]. We assume, these competitive bindings to be different microstates of an ensemble, particularly canonical ensemble at constant temperature (physiological temperature 300 K), which all are the binding sites available for any particular miRNA in that system. We here estimated average binding free-energy from these microstates using formula of ensemble average, considering

Table 2
Free energy of binding and duplex formation between mRNAs or ceRNAs with the shared miRNAs considering the presence of all ceRNAs, transcripts of interests and shared miRNAs together.

Transcripts of interest	ceRNAs	Average Energy of duplex formation (kcal)	Average Weighted Energy of duplex formation (kcal)	Cancer type
CDKN1A		−6.191092	−0.00078	CML
	E2F2	−4.44923	−5.9E−07	
	SMAD4	−8.15643	−9.03E−06	
	ACVR1B	−6.71897	−37.8982	
	AKT3	−0.081091	−9.9E−28	
ABL1		−6.43593	−2.56652E−11	
	GRB2	−20.5323	−36.5983791	
BTN2A1		−2.682107776	−1.16577E−06	
	CBL	−19.4389	−38.5992	
	ACVR1C	−8.985299142	−3.26272E−09	
CNST		−4.639333444	−0.023085889	
	KRAS	−2.91861	−0.027454817	
	TGFBR2	−14.0578	−26.9331	
	SOS1	−8.39901	−0.190691025	
ENPP1		−14.94923401	−37.4957	
	NRAS	−8.65441	−3.12561E−08	
	MDM2	−5.93971	−1.5063E−05	
SYNM		−4.437655189	−20.70884397	
	CHUK	−0.728279528	−5.51822E−18	
	SMAD4	−10.81648937	−0.03703	
	CDKN1A	−15.7745	−11.0316	
	MAPK1	−6.952535053	−0.04622	
CLOCK		−7.12569	−15.59736773	AML
	SOS1	−10.3059	−12.68859181	
	MAPK1	−16.1245	−1.040573562	
SUZ12		−4.00811	−2.17364E−05	
	PIK3CA	−2.67965	−0.001108644	
	RPS6KB1	−12.2865	−0.162216067	
	SOS1	−13.6779	−29.09540897	

probabilities of binding to any particular site, which is $\exp(-\Delta G/k_B T)/Z$ [41], where Z is the partition function and is defined as

$$Z_{miR} = \sum_{i=1}^{N} \exp(-\Delta G_i/k_B T) \quad (1)$$

The summation runs over all possible N microstates, which are binding sites of an miRNA on all the mRNAs belonging to a sub-ceRNET. Thus, the average free energy of binding of $miRNA_x$ to $mRNA_a$ is calculated as

$$\Delta G_{miR}^{trn} = \frac{\sum_{i=1}^{n_c} \Delta G_i \exp(-\Delta G_i/k_B T)}{Z_{miR}} \quad (2)$$

n_c is the number of sites on an $mRNA_a$ where the specific $miRNA_x$ can bind. ΔG_{miRtrn} values for different miRNA:mRNA binding energies are given in Supplementary Table S4. Please note that the N for calculation of partition function Z is much larger (consisting of all site for an miRNA on all the mRNAs in a sub-ceRNET) than the value n_c used for calculation of average free energy (all sites for an miRNA on a particular mRNA). The binding free energy values are seen to be widely different for different miRNAs:mRNA systems. As for example, the binding energy obtained by the above statistical mechanical method between *CDKN1A* and hsa-miR-1227-5p is found to be −31.54 kcal/mol while that between *CDKN1A* and hsa-let-7e-5p is −0.03 kcal/mol, indicating huge difference in average energies of interactions between mRNA and miRNAs.

3.4. Evaluating the competitive cross-talking within a sub-ceRNET through statistical thermodynamics

Next, we aimed to figure out the significant cross-talking tracks or the important ceRNA members in the interior of individual sub-ceRNETs. In point of fact, here, we aimed to evaluate the extent of competition of the ceRNA members with the 'Transcripts of Interest' for all shared miRNAs in a ceRNA sub-network. In our analysis, free energy for binding and duplex formation between mRNAs or ceRNAs and shared miRNAs showed a variation among ceRNA members in a ceRNA set of a certain transcript of interest.

In a cell, there are multiple shared miRNAs present at the same time, and hence, a second level of competition arises within the pool of shared miRNAs and mRNAs (or ceRNAs). We have calculated the average binding free-energies within the pool of miRNAs and mRNAs from the above free-energies using similar statistical mechanical averaging technique.

When multiple miRNAs and mRNA transcripts of a sub-ceRNET are present, the miRNAs can bind to many mRNAs to repress them. Such repression would also depend on competitive binding between the miRNAs and transcripts. Hence, we carried out a second level of statistical mechanical averaging considering the free energies derived and given in Supplementary Table S4.

Considering the presence of limited pool of miRNAs, which is rationally a requisite for ceRNA competition, here we hypothesize that, if a given miRNA binds to a transcript, it cannot bind to another transcript at the same time and hence, a competition exists for which transcript would be repressed by the shared miRNAs. Therefore, the partition function includes contribution for all the shared miRNAs and all the transcripts in a sub-ceRNET and can be defined as

$$Z = \sum_{n_{miR} * n_{trn}} \exp(-\Delta G_i/k_B T) \quad (3)$$

where n_{miR} is number of miRNAs and n_{trn} is number of mRNA transcripts of a sub-ceRNET. The suppression of a transcript would depend on average free energy for binding of all miRNAs to that transcript. Such average free energy is calculated as

$$\Delta G_f = \frac{\sum_i \Delta G_i \exp(-\Delta G_i/k_B T)}{Z} \quad (4)$$

here i varies from 1 to number of miRNA which has binding site to a particular transcript, while the Z is calculated considering Eq. (3).

Henceforth, considering the scenario that, if an miRNA binds to a target, there would not be more of that miRNA in the system to bind with another target molecule in that sub-ceRNET, we calculated simple average as well as statistical mechanical average of free-energy values and provided in Table 2.

We found from the values in Table 2 that average free-energy between the miRNAs and the mRNA for *CDKN1A* is negligible (−0.00078) as compared to the free-energy between the miRNAs and mRNA for *ACVR1B* (−37.8982). This indicates that in CML system, within the *CDKN1A* sub-ceRNET, *ACVR1B* sponges most of the miRNAs as it has best statistical mechanical average energy. This possibly indicates that *CDKN1A* would most probably be highly expressed while *ACVR1B* would exhibit a strong cross-talk via miRNA sponging and subsequently be suppressed by the shared miRNAs. Similarly, cross-talk between *ABL1* and *GRB2*; *BTN2A1* and *CBL*; *CNST* and *TGFBR2* are maximum in CML from the perspective of miRNA sponging effect. The cross-talk in the *ENPP1* and *SYNM* sub-ceRNETs, however, are of different kind, where the ceRNAs do not have much sponging effect as the free-energies of the shared miRNAs are small for both *NRAS*, *MDM2* (for *ENPP1* cluster) and *CHUK*, *SMAD4*, *CDKN1A*, *MAPK1* (in *SYNM* cluster). Nonetheless, substantial difference in binding energy is observed between 'Transcripts of Interest' and ceRNA partners which in turn shows a significant cross-talking among them. In case of AML systems cross-talk or competition between *CLOCK, SOS1* and *MAPK1* was observed in *CLOCK* sub-ceRNET. Again, cross-talk between *SUZ12* and *SOS1* are maximum in *SUZ12* cluster where *SOS1* shows a high miRNA sponging.

3.5. Relevance of the major ceRNA cross-talkers in CML and AML systems

Nodes communicating across the significant cross-talking edges in

the sub-ceRNETs can serve as biomarkers and possible drug targets in human Chronic and Acute Myeloid Leukemia. We next sought to find the importance of these nodes in regulating carcinoma.

3.5.1. CML system

3.5.1.1. CDKN1A sub-ceRNET. In a cancerous cell *CDKN1A* with the help of p21 arrests cell cycle progression and allows cells to either repair DNA damage or undergo apoptosis [45]. *SMAD4*, a ceRNA in this sub-ceRNET is important for transcriptional activation and inhibition of cell growth after *TGF-β1* stimulation. Mutation of *SMAD4* gene in leukemia blocks the anti-proliferative effect of *TGF-β* [46]. *ACVR1B* is mainly involved in cell survival, cell proliferation, tumor growth, tumor invasion and blood vessel expansion [47]. *E2F2*, another ceRNA partner, plays a crucial role in the control of cell cycle and action of tumor suppressor proteins, specifically to retinoblastoma protein pRB [48]. The last ceRNA in this set, *AKT3* is involved in a wide variety of biological processes including cell proliferation, differentiation, apoptosis and tumorigenesis [49].

3.5.1.2. ABL1 sub-ceRNET. BCR-ABL1 fusion protein is the most relevant signature of CML. Cells harboring this fusion protein were shown to be imatinib-sensitive, however, additional mutations in *ABL1* conferred resistance to imatinib [50]. *GRB2*, the cross-talking ceRNA in this sub-ceRNET, contains SH2 domain which binds to phosphorylated tyrosine residue 177 on *BCR*. Phosphorylation of this regulatory domains of *BCR* is mediated through *ABL* leading to *BCR-ABL*-mediated leukemogenesis and *RAS/MAPK* activation [51].

3.5.1.3. BTN2A1 sub-ceRNET. BTN2A1 encoded protein is an integral plasma membrane protein. Proteomic analysis from membrane samples in K562 CML cells showed enrichment for integral membrane proteins [52] which might be involved in various signaling pathways in CML. A decrease in *CBL* (a ceRNA of *BTN2A1*) expression leads to tyrosine kinase over-expression and activation sufficient to induce resistance to nilotinib in CML cells [53]. The other cross-talker in this sub-ceRNET, *ACVR1C* is a type I receptor for the *TGF-β* family of signaling molecules which phosphorylate cytoplasmic *SMAD* transcription factors. These then translocate to the nucleus and regulates expression of genes involved in tumor growth and invasion [54].

3.5.1.4. CNST sub-ceRNET. Consortin (*CNST*) is an integral membrane protein which acts as a binding partner of connexins, the building blocks of gap junctions [55], are involved in leukemogenesis and are important for chemo sensitivity [56]. *KRAS*, a ceRNA in this set, plays an important role in promoting oncogenic events by inducing transcriptional silencing of tumor suppressor genes [57]. Again, reduced expression of *TGFBR2*, member of this set, in CML cells and leukemia cell lines plays a role in the initiation and/or maintenance of the disease state [58]. *SOS1*, belongs to the set of genes upregulated by *BCR/ABL* kinase and also acts as a ceRNA interactor of *CNST* [59].

3.5.1.5. ENPP1 sub-ceRNET. ENPP1 was not found to be associated with leukemia pathogenesis, however, by acting upstream of *E2F1*, *ENPP1* was seen to be indispensable for the maintenance of glioblastoma stem-like cells (GSCs) in vitro and hence required to keep GSCs in an undifferentiated, proliferative state [60]. Studies suggested that, addition of oncogenic *NRAS* (NRASG12D) to a vector containing a *BCR/ABLY177F* mutant “rescues” the CML phenotype rapidly and efficiently in mouse bone marrow transduction or transplantation system [61]. In a study on *MDM2*, a ceRNA of this ceRNET, it was observed that, the carriers of the *MDM2* SNP309 G/G genotype were associated with increased susceptibility of CML [62].

3.5.1.6. SYNM sub-ceRNET. It has been observed that, association of *SYNM* with the dystrophin complex and its cancer-specific expression loss, could probably interrupt the conjunction between the extracellular matrix and intermediate filament network of myoepithelial breast cells. It consequently decreases cell adhesion, supports metastatic spread and leads to tumor progression [63]. Studies on imatinib-resistant kinome profile in CML revealed upregulation of *CHUK* (*IKKα-NF-κB* pathway) which is involved in cell growth, anti-apoptosis and stress signaling [64]. The *MAPK1* and TGF-β pathways were seen to play critical roles in cell development and cell cycle regulation, as well as in tumorigenesis and metastasis. Though in the absence of cellular transformation, these pathways function in opposition to one another, in cancer system they act as ‘partners in crime’ as their concomitant activation drives proliferation, survival and differentiation [65]. *SMAD4*, another ceRNA in this sub-ceRNET, is also associated with the TGF-β pathway as mutation of this gene in leukemia blocks the anti-proliferative effect of *TGF-β* [46].

3.5.2. AML system

3.5.2.1. CLOCK sub-ceRNET. CLOCK is a component of canonical circadian pathway, disruption of which produces impaired proliferation, enhanced myeloid differentiation, and depletion of leukemia stem cells (LSCs) in AML [66]. *SOS1,* a ceRNA interactor in this sub-ceRNET, is a *Ras guanine nucleotide exchange factor (GEF).* Activation of *SOS1* mediates Growth factor activation of Ras and consequently may play a role in myeloid cell signaling growth regulation pathways [67]. Another ceRNA in this set is *MAPK1*. The *MAPK1*-pathway is often aberrantly activated in AML and is known to contribute to myeloid leukemogenesis [68] by promoting cellular proliferation and inhibiting apoptosis [69].

3.5.2.2. SUZ12 sub-ceRNET. Mutation in *SUZ12* yields abnormalities in AML and MDS/MPN patients [70]. Depletion of *SUZ12* was observed to be associated with an inhibition of colony growth but not apoptosis of the cultured AML cells [71]. Aberrant expression of *PIK3CA*, ceRNA in this set, which also belongs to *PI3K/Akt/mTOR* signaling, has been implicated in many human cancers, including acute myelogenous leukemia (AML) as *PI3K/Akt/mTOR* signaling plays a central role in cell proliferation, growth, and survival under physiological conditions [72]. Activation of *RPS6KB1* drives glycolysis in leukemia cells, as a target for counteracting glucose-dependent survival induced by BCR-ABL [73]. *SOS1*, ceRNA in the previous sub-ceRNET, again appears in this set too, whose implication in AML progression has been mentioned previously.

4. Discussion and further directions

ceRNA language based on the presence of MREs does not count on the protein-encoding genetic blueprint aspects of an mRNA, rather directs it to communicate to the other components of the transcriptome including transcribed pseudogenes, long non-coding RNAs (lncRNA) and circular RNAs [4]. ceRNAs emerging as a novel and intriguing player of gene regulation can thus co-regulate each other and add a new layer of complexity in human disease progression [74–76].

In this study we depicted the molecular competition encapsulated in a number of ceRNA functional modules relevant in Chronic and Acute Myeloid Leukemia and tried to shed light on the mainstream research on how the sequence rivalry ends up in a prevalent form of gene regulation. Paired miRNA and mRNA expression from patient sample data were integrated with bioinformatics target prediction to design CML and AML specific ceRNA networks. A number of ceRNA sub-networks pertinent to CML and AML biology were retrieved through functional enrichment analysis and henceforth the co-expression pattern of the ceRNAs in both CML and AML cell line was validated through Q-PCR analysis. We evaluated the free energy of binding and duplex formation and consequently the probability of the ceRNAs in each module to be expressed or to get repressed by the shared miRNA molecules through the knowledge of miRNA binding pattern in different MREs present on transcripts. Delving deeper, through an approach of statistical

thermodynamics, we provided an indication of the influential ceRNA molecules within each sub-ceRNET which can substantially modulate the 'Transcripts of Interest'. While evaluating the probability of a ceRNA to execute the miRNA-sponging effect or to attain the statistical mechanical energy of binding and duplex formation between an miRNA and an mRNA, we followed a two way possibility in choosing the Partition function. We considered two situations 1) excess population of all miRNAs in the cell, so binding of a particular miRNA with a transcript will not hinder the availability of other miRNA molecules of same sequence to bind to another transcript of any other gene, 2) limited number of miRNA pool, so miRNA binding to a particular transcript, would inhibit that specific miRNA to bind to another transcript. However we feel the possibility of the second one, i.e. occurrence of limited concentration of miRNA in cell with its target transcripts, is biologically more meaningful.

While retrieving the functional modules participating in the cancer biology of both type of myeloid leukemia, we had to restrict our study only in the Normal and Blastic stages due to unavailability of miRNA expression data in Chronic or Accelerated phases. The ceRNA neighborhood was expected to be changed while shifting from Normal to Cancer system [13] which was again reflected in the altered ceRNA sub-ceRNET of *CDKN1A* transcript across CML and Normal system. In the latter one the *CDKN1A* ceRNA set contains a totally different set of neighbors (*RASGRF1*, *DEDD*, *PLAGL2*, *RUNX3 and TNFSF10*). Hereafter, we, in this study aimed to identify the ceRNA molecules having maximal potentiality to modulate the 'Transcripts of Interest' in the selected leukemia sub-ceRNETs which essentially represent the altered ceRNA landscape in cancer system. Our computational analysis yielded certain particular ceRNAs to exhibit highest efficacy in miRNA sponging effect in the selected ceRNA functional modules of the two myeloid leukemia.

Assessing the major cross-talking ceRNAs indeed reveals the important regulators of a number of genes at 'transcript level'. *CDKN1A* a transcript associated in cell proliferation exhibits a strong cross-talk with its co-expressed ceRNA partner *ACVR1B* which is again involved in tumor growth and invasion. Also, *ABL1* transcript playing an imperative part in making of the *BCR-ABL1* fusion protein, a hallmark in CML pathogenesis, cross-talks with *GRB2* leading to *BCR-ABL*-mediated leukemogenesis and *RAS/MAPK* activation. On the other hand, in AML, the Clock Circadian Regulator displays a sibling rivalry with its ceRNA interactors *SOS1* and *MAPK1* which have implications in growth regulatory pathways and promoting cellular proliferation. Moreover, *SUZ12*, a chromatin modifier, interact with ceRNAs contributing to the *PI3K/Akt/mTOR* network. The *PI3K/Akt/mTOR* signaling axis has been observed to be constitutively active in AML and thereby affects survival, proliferation and drug-resistance of leukemic cells. Thus, this renders its pivotal role as a validated target for innovative cancer therapy. 'Turning on' or 'turning off' of the major ceRNA or cross-talkers through siRNA knock down may in turn modulate the expression and thereby function of the oncogenes or tumor suppressors concerned.

It is also noteworthy here that, AML is a clinically incredibly heterogeneous disease characterized by an assembly of chromosomal abnormalities and gene mutations. Thus the ceRNA network characteristics may get deviated as it entirely depends on the level of mRNA and miRNA expression in each sub-type. For instance, a specific sub-ceRNET for FLT-1, which was observed to regulate the migration and possibly the proliferation of the malignant plasma cells [77], was obtained in our study and may change its neighborhood ceRNAs if it would be studied in a different sub-type with a different landscape of transcript expression level.

We here conclude that ceRNA networks, resulted from the cross-talking affairs of protein coding and non-coding transcripts, open an important stratum of research on gene regulation in human cancer, its development and prognosis. We positively anticipate that probing further into the dynamics of miRNA sponging and degradation of the ceRNA regulators will be worthy to unveil gene regulatory mechanisms for tumor suppressors and oncogenes in human Chronic and Acute Myelogenous Leukemia. Further study on these ceRNA regulators in the dynamics of CML and AML specific ceRNA network will definitely be worthy in the etiology of human Chronic and Acute Myeloid Leukemia.

4.1. Limitations of the study

We constructed ceRNA networks for CML and AML system based on only patient sample data on mRNA expression from one study. We admit that, considering more study on CML and AML patient sample data might strengthen and affirm our results. On the other hand, though we executed our ceRNA analysis on patient sample data, due to ethical hindrance in procuring clinical samples, we had to perform the Q-PCR analysis on CML and AML cell lines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2018.06.002.

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Conflict of interest

The authors have no conflicts of interest to declare.

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