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MicroRNAs that affect the Fanconi Anemia/BRCA pathway are downregulated in imatinib-resistant chronic myeloid leukemia patients without detectable *BCR-ABL* kinase domain mutations

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ABSTRACT

Chronic myeloid leukemia (CML) patients who do not achieve landmark responses following treatment with imatinib mesylate (IM) are considered IM-resistant. Although IM-resistance can be due to *BCR-ABL* kinase domain (KD) mutations, many IM-resistant patients do not have detectable *BCR-ABL* KD mutations. MicroRNAs (miRNAs) are short non-coding RNAs that control gene expression. To investigate the role of miRNAs in IM-resistance, we recruited 8 chronic phase CML patients with IM-resistance who tested negative for *BCR-ABL* KD mutations and 2 healthy normal controls. Using miRNA sequencing, we identified 54 differentially expressed miRNAs; 43 of them downregulated. The 3 most differentially downregulated miRNAs were miR-146a-5p, miR-99b-5p and miR-151a-5p. Using real-time quantitative reverse transcriptase-polymerase chain reaction, the expression patterns of the 3 miRNAs were validated on the same cohort of 8 patients in addition to 3 other IM-resistant CML patients. *In-silico* analysis showed that the predicted gene targets are *ATRIP, ATR, WDR48, RAD51C* and *FANCA* genes which are involved in the Fanconi Anemia/BRCA pathway. This pathway regulates DNA damage response (DDR) and influences disease response to chemotherapy. Thus it is conceivable that DDR constitutes a key component in IM-resistance. Further research is needed to elucidate miRNA modulation of the predicted gene targets.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloid neoplasm caused by the *BCR-ABL* fusion gene, a product of the chromosomal translocation t (9:22) which results in the Philadelphia (Ph) chromosome. This fusion gene encodes the BCR-ABL oncoprotein, a constitutively active tyrosine kinase that causes dysregulated cellular proliferation and apoptosis resistance via interference in downstream signalling pathways. Current standard management of patients who have CML is with *BCR-ABL* inhibition with tyrosine kinase inhibitors (TKIs) such as imatinib mesylate (IM). Resistance to IM is an emerging issue [1]. Failure to achieve established landmark responses despite IM therapy constitutes treatment failure and the patient is deemed resistant to IM. When this occurs, the patient is tested for possible kinase domain (KD) mutations on the *BCR-ABL* fusion gene and possibly be treated with an alternative TKI. KD mutations of *BCR-ABL* are responsible for approximately 40% of all cases of resistance [2]. Thus, in the majority of patients who failed TKIs, no KD mutations are detected. Patients who are resistant to all commercially-available TKIs with no detectable *BCR-ABL* KD mutations have poor prognosis. Due to the increasing prevalence of CML and the availability of more potent TKIs, this group of patients represents an unmet medical need.

MicroRNAs (miRNAs) belong to one of the classes of non-coding RNAs which are functional RNAs that do not translate into protein. miRNAs are in the range of 19–22 nucleotides long and transcribed from pri-miRNAs which are first processed into pre-miRNAs by Drosha, an intranuclearRNase III enzyme. Pre-miRNAs are then exported to the cytoplasm where Dicer, another RNAse III enzyme cleaves them to give rise to mature miRNAs. MiRNAs play an important role in hemato-poeisis from apoptosis to cell differentiation regulation [3]. The role of miRNAs in the pathogenesis of CML is established, and has expanded the role of *BCR-ABL* [4–7]. It has also been shown that miRNA profile

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Table 1
Patient Characteristics.

Patient	Age	Sex	Switched to Nilotinib	Duration of Imatinib (months)	Duration of Nilotinib (months)	Time to Hematologic Remission (months)	Best Cytogenetic Response	Time to Best Cytogenetic Response (months)	Sokal Score at diagnosis
1	24	M	N	61.5	–	2.5	Minor	36.57	1.52
2	55	M	Y	56.94	11.73	1.41	Partial	53.55	0.67
3	65	F	N	21.09	–	10.97	Minor	34.96	1.37
4	55	M	Y	26.35	10.58	0.66	Complete	36.90	1.12
5	28	M	N	120.05	–	3.22	Complete	21.62	0.48
6	52	F	N	28.88	–	2.3	Minor	10.15	1.3
7	44	M	Y	54.31	26.45	2.04	Complete	56.61	1.57
8	30	F	Y	89.26	53.16	39.79	Partial	134.93	0.55

Complete cytogenetic response (CCyR) − no Ph-positive metaphases.
Partial cytogenetic response (PCyR) − 1–34% of cells have Ph-positive metaphases.
Major cytogenetic response (MCyR)- 0–35% of cells have Ph-positive metaphases (Complete + Partial).
Minor cytogenetic response- > 35% Ph-positive metaphases.

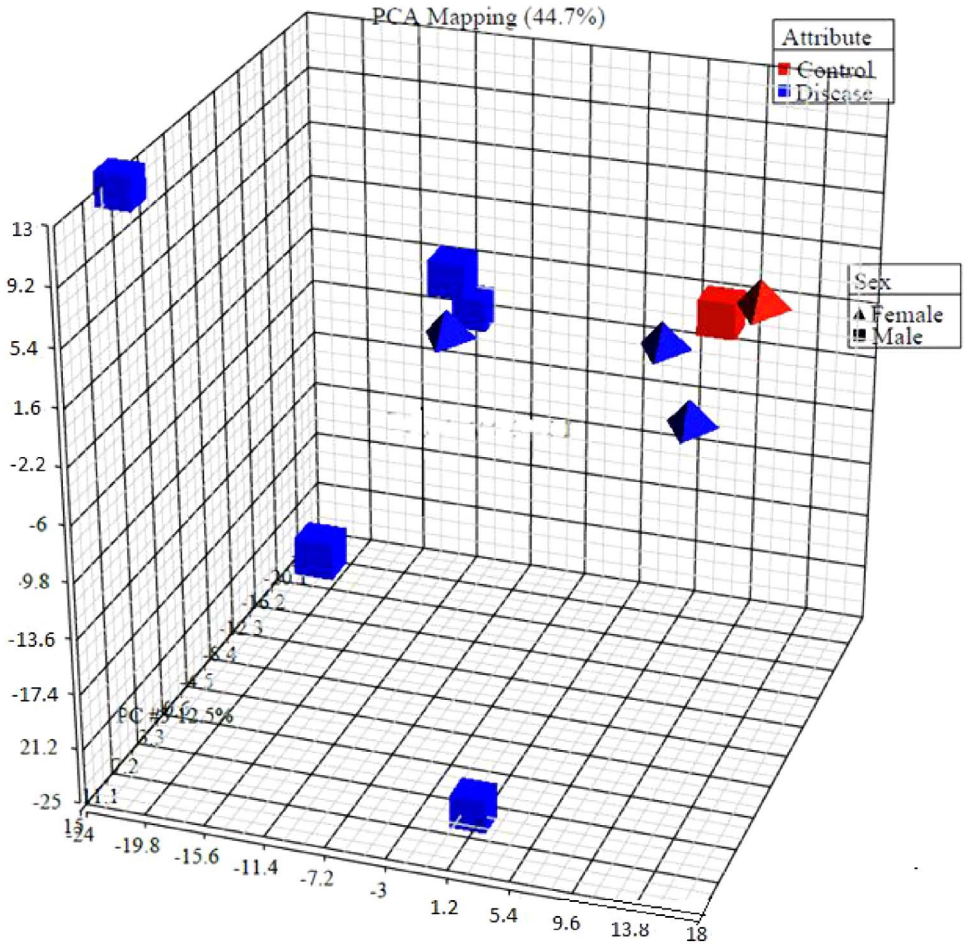

Fig. 1. 3-Dimensional Principal Component Analysis (PCA) mapping showing distinct clustering of IM-resistant CML and normal controls.

undergoes dynamic changes following IM therapy [8,9].

We hypothesize that miRNA dysregulation also plays a role in IM-resistance in which no *BCR-ABL* KD mutations are identified. Next generation sequencing (NGS) can detect the expression levels of each miRNA in the miRNome, and is thus an effective and accurate approach for evaluation of global miRNA expression levels. To the best of our knowledge, NGS of the miRNome of CML patients who are IM-resistant has not previously been performed, and such an analysis may provide insight into the molecular mechanisms of this phenomenon.

2. Materials and methods

We enrolled 8 patients (5 Malays, 2 Chinese and 1 Indian) from the National University of Malaysia Medical Centre (UKMMC) diagnosed with CML in chronic phase, demonstrated primary resistance to IM as described in the National Comprehensive Cancer Network (NCCN) 2015 guidelines [10] and tested negative for *BCR-ABL* KD mutations. Briefly, mutation analyses were undertaken using denaturing high performance liquid chromatography (dHPLC). The PCR products of samples that showed altered dHPLC profile were then directly sequenced with both forward and reverse primers after purification.

Table 2a
Significantly downregulated miRNAs in IM-resistant CML patients compared to normal controls.

MiRNAs that are downregulated			
	Marker_id	p-value (Disease vs. Control)	Fold Change (Disease vs. Control)
1	hsa-miR-146a-5p	3.79E-06	−6.22324
2	hsa-miR-7849-3p	4.26E-06	−33.8249
3	hsa-miR-99b-5p	0.000113203	−2.94754
4	hsa-miR-151a-5p	0.000203563	−4.5282
5	hsa-miR-10a-5p	0.000322269	−3.56621
6	hsa-miR-151a-3p	0.000392012	−3.74401
7	hsa-miR-6810-3p	0.000702386	−8.33223
8	hsa-miR-143-3p	0.00118648	−2.52804
9	hsa-miR-335-3p	0.00122188	−2.41596
10	hsa-miR-584-5p	0.0014285	−3.28754
11	hsa-miR-1299	0.00147806	−15.1559
12	hsa-miR-125a-5p	0.00214055	−2.15144
13	hsa-miR-486-5p	0.0021741	−8.50905
14	hsa-miR-486-5p	0.00218336	−8.48646
15	hsa-miR-4446-3p	0.00222185	−3.25009
16	hsa-miR-5010-5p	0.00228652	−4.0877
17	hsa-miR-320a	0.00270866	−12.0229
18	hsa-miR-4742-5p	0.00338246	−4.27477
19	hsa-miR-4433-3p	0.00343567	−5.15747
20	hsa-miR-4433b-5p	0.00343567	−5.15747
21	hsa-miR-4667-5p	0.00905594	−8.16113
22	hsa-miR-6782-3p	0.00905594	−8.16113
23	hsa-miR-5683	0.00905594	−8.16113
24	hsa-miR-2114-3p	0.00930315	−6.62493
25	hsa-miR-379-5p	0.0120323	−10.9135
26	hsa-miR-4639-5p	0.0120892	−7.75461
27	hsa-miR-139-3p	0.0139587	−4.35767
28	hsa-miR-493-3p	0.0144447	−3.22336
29	hsa-miR-362-5p	0.0163726	−2.02148
30	hsa-miR-30a-3p	0.0177087	−3.00015
31	hsa-miR-190a-5p	0.0213447	−7.20479
32	hsa-miR-3661	0.0246618	−4.10177
33	hsa-miR-4732-3p	0.0265198	−12.3984
34	hsa-miR-338-3p	0.0302762	−36.3675
35	hsa-miR-6511b-3p	0.0316625	−2.50349
36	hsa-miR-6511b-3p	0.0316625	−2.50349
37	hsa-let-7e-5p	0.03507	−4.47001
38	hsa-miR-3688-5p	0.0352901	−5605.43
39	hsa-miR-3688-5p	0.0352901	−5605.43
40	hsa-miR-6813-5p	0.0353892	−2.85137
41	hsa-miR-99a-5p	0.0377892	−2.40474
42	hsa-miR-451a	0.042691	−6.75015
43	hsa-miR-5588-5p	0.0458527	−4.24512

Table 2b
Significantly upregulated miRNAs in IM-resistant CML patients compared to normal controls.

MiRNAs that are upregulated			
	Marker_id	p-value (Disease vs. Control)	Fold Change (Disease vs. Control)
1	hsa-miR-6502-5p	0.00986512	2.53729
2	hsa-miR-642a-5p	0.0137339	3.0256
3	hsa-miR-589-3p	0.0143791	2.59306
4	hsa-miR-27a-5p	0.0167331	2.27191
5	hsa-miR-378c	0.0185071	2.36526
6	hsa-miR-1306-5p	0.0195071	2.16772
7	hsa-miR-3607-3p	0.0238666	57.483
8	hsa-miR-26b-3p	0.0320052	2.21988
9	hsa-miR-2277-5p	0.0402193	2.70147
10	hsa-miR-4645-3p	0.0441672	6.29132
11	hsa-miR-660-5p	0.0484582	2.18455

Further details are described by Elias et al. [11]. Median age of the patients was 48 years (range 24–65 years) (Table 1). Most patients began IM therapy shortly after diagnosis and the follow-up time ranged from 2 to 73 months (median 46 months). One male and one female healthy volunteer constitute the normal control group. Informed consents were obtained from the patients and normal controls. Study approval was obtained from the Ethics Committee, UKMMC. All methods employed in this study were in accordance with the Declaration of Helsinki.

2.1. Separation of leucocytes

Approximately 10 mL of peripheral blood were collected from the CML patients and normal controls. The samples were treated with Red Blood Cell Lysis Buffer (eBioscience, San Diego, USA) for 10 min on ice followed by centrifugation at 500 x *g* for 15 min at 4 °C to separate the leucocytes.

2.2. RNA extraction, library preparation and sequencing

Generally, the NGS sequencing of miRNAs has been described [12]. Total RNA was extracted using miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's guide. The concentration, purity and integrity of total RNA were assessed using Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Wilmington, USA), NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and Agilent 2100 Bioanalyzer system with RNA 6000 Nano kit (Agilent Technologies, Santa Clara, USA) respectively. Only those samples with purity (A260/A280 ratio) of between 1.8–2.1 and RNA Integrity Number (RIN) of ≥8 were chosen for small RNA library preparation. One microgram total RNA was used as a template to construct cDNA library using TruSeq™ Small RNA Libary Preparation Kit (Illumina, San Diego, USA) as per manufacturer's instructions. Total RNA was ligated with 3′- and 5′- adapter followed by reverse transcription and amplification in which specific index sequences were incorporated. The libraries were then purified by size selection (140–160 bp) on 6% polyacrylamide gel electrophoresis (PAGE) gel followed by validation and quantification using Qubit DNA HS Assay (Thermo Fisher Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer High Sensitivity DNA chip (Agilent Technologies, Santa Clara, USA). The libraries were pooled and sequenced on an Illumina Miseq sequencer (Illumina, San Diego, USA) by paired-end miRNA sequencing (miRNA-seq).

2.3. MiRNA expression profiling analysis

Small RNA reads (15–35 *nt*) were sequenced and extracted to comprehensively survey the microRNAome. The analysis was performed using the following steps: 1) Adaptor sequences were trimmed using FASTQ-MCF program [13]. 2) Low quality reads (below Q33 phred score) and reads with ambiguous bases (other than A, C, G and T) were discarded. 3) Subsequent removal of rRNA, tRNA and snoRNA based on Rfam (Release 12.0) [14] and GtRNAdb [15] databases were performed and the remaining reads were designated as clean reads. 4) BAM files were generated by mapping the clean reads to human genome (HG19) using the Bowtie program [16] with the option of reporting all alignments per read. Only reads within the sequence length of 15-35 *nt* that were mapped (with the allowance of maximum 3 mismatches) to the reference genome were retained and subjected to downstream analysis using Partek® Genomics Suite® software, version 6.6, St. Louis, MO, USA.

The miRNA expression levels were compared between patients and normal controls to detect the differentially expressed miRNAs. The data was first normalized with reads per kilobase per million (RPKM) method to obtain the expression of transcripts per million [17]. Principal component analysis (PCA) was applied using normalized data to visualize sample plots among biological replicates. The statistical

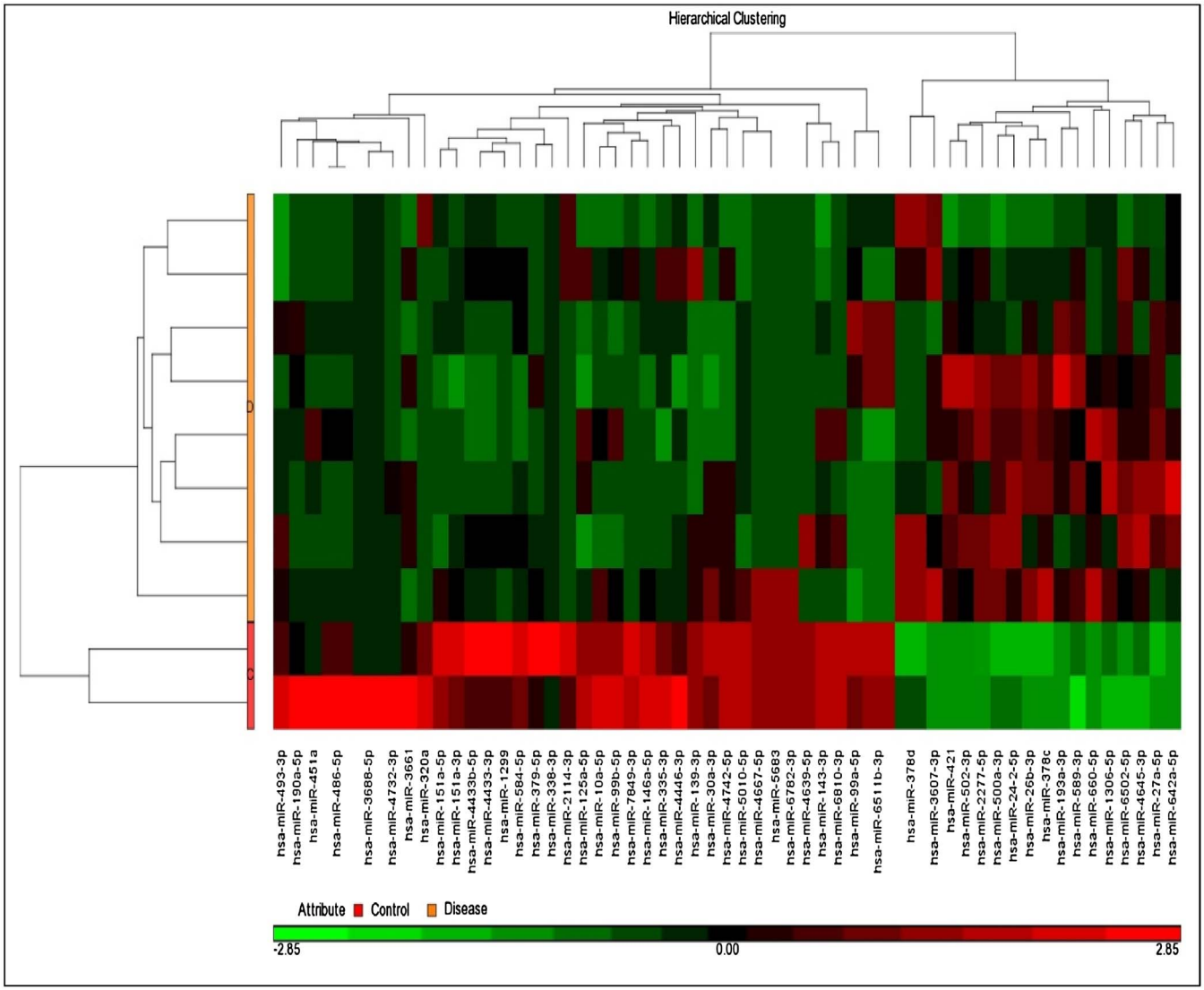

Fig. 2. Unsupervised hierarchical clustering of miRNA showing upregulated (red) and downregulated (green) miRNAs in IM-resistant CML patients and normal controls. IM-resistant CML patients demonstrated a distinct miRNA expression profile compared to normal controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

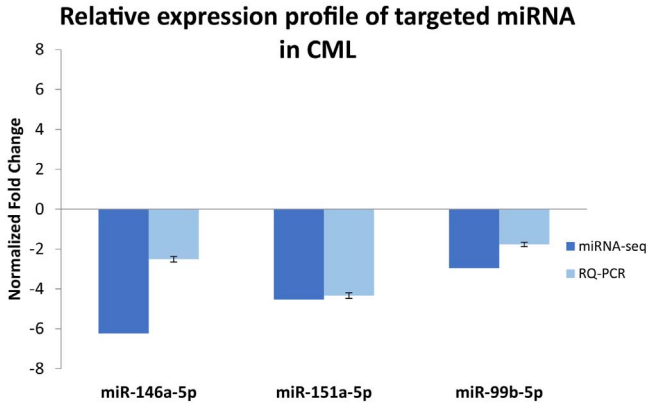

Fig. 3. Relative expression profiles by miRNA-seq and RQ-PCR. The miRNA-seq results for miR-146a-5p, miR-151a-5p and miR-99b-5p were successfully validated using RQ-PCR.

analysis of the sequencing data was performed using Analysis of Variance (ANOVA) test and only miRNAs with $p \leq 0.05$ and fold change (FC) ≥ 2 were considered to be significant. Hierarchical clustering was performed on the differentially expressed miRNAs to group them according to similarity defined by correlation coefficient.

2.4. Validation of miRNA-seq results

In order to validate the miRNA-seq data, we selected the 3 most differentially expressed (downregulated) miRNAs: miR-146a-5p, miR-99b-5p and miR-151a-5p for further validation by real-time quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) using miScript PCR System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A total of 200 ng of total RNA from the same 10 samples used in sequencing with 3 additional samples from a different patient cohort were processed using miScript II RT kit (Qiagen, Hilden, Germany). The assays were run with miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) in triplicates on the CFX96™ Real-Time Detection System (Bio Rad, California, USA). The PCR was performed under the following conditions: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. The miR-191 was used as endogenous control as it showed constant expression in the sequencing data. Non-template control (NTC) and miRNA reverse transcriptase control (miRTC) were also prepared to detect possible contaminations. The expression data was then analyzed by relative

Table 3a
TargetScan predicted interactions for miR-146a-5p and their putative gene targets.

TargetScan Predicted Interactions for miR-146a-5p			
	Gene Name	Gene Ensembl id	Context Score
1	*WDR47*	ENSG00000085433	−0.401
2	*RHOBTB3*	ENSG00000164292	−0.654
3	*ZBTB2*	ENSG00000181472	−0.569
4	*COPS8*	ENSG00000198612	−0.553
5	*TIMELESS*	ENSG00000111602	−0.624
6	*ATAD1*	ENSG00000138138	−0.492
7	*RHOXF2*	ENSG00000131721	−0.432
8	*ITCH*	ENSG00000078747	−0.466
9	*LCOR*	ENSG00000196233	−0.473
10	*BHLHE41*	ENSG00000123095	−0.516
11	*CSPP1*	ENSG00000104218	−0.604
12	*LCA5L*	ENSG00000157578	−0.589
13	*SLC38A9*	ENSG00000177058	−0.427
14	*ZNF253*	ENSG00000256771	−0.814
15	*CTAGE4*	ENSG00000225932	−0.483
16	*CTAGE8*	ENSG00000244693	−0.483
17	*C9orf72*	ENSG00000147894	−0.418
18	*PRR9*	ENSG00000203783	−0.402
19	*IGSF1*	ENSG00000147255	−0.557
20	*ATRIP*	ENSG00000164053	−0.449
21	*TRAF6*	ENSG00000175104	−0.738
22	*SRSF6*	ENSG00000124193	−0.514
23	*TMEM185B*	ENSG00000226479	−0.49
24	*ZNF506*	ENSG00000081665	−0.544
25	*BCORL1*	ENSG00000085185	−0.404
26	*ST7L*	ENSG00000007341	−0.421
27	*CTAGE9*	ENSG00000236761	−0.476
28	*C15orf57*	ENSG00000128891	−0.42
29	*BZW1*	ENSG00000082153	−0.426
30	*ACKR2*	ENSG00000144648	−0.484
31	*SIRPB1*	ENSG00000101307	−0.498
32	*ZNF652*	ENSG00000198740	−0.568
33	*IRAK1*	ENSG00000184216	−0.7
34	*PSMF1*	ENSG00000125818	−0.518
35	*PHKB*	ENSG00000102893	−0.51
36	*REEP5*	ENSG00000129625	−0.421
37	*ATR*	ENSG00000175054	−0.422
38	*ZDHHC13*	ENSG00000177054	−0.408
39	*ZNF486*	ENSG00000256229	−0.589

quantification ($2^{-\Delta\Delta CT}$) using CFX Manager™ Software v3.1 (Bio Rad, California, USA).

2.5. Prediction of target genes for differentially expressed miRNAs

To identify possible roles for the above identified microRNAs in IM-resistant CML, we investigated *in silico* for microRNA binding sites predicted by TargetScan algorithm using the miRPath v.3 (http://www.microrna.gr/miRPathv3/) [18]. We then sought to merge the results (p-value threshold 0.05; TargetScan context score less than −0.4) to obtain predicted pathways influenced by the putative gene targets using the KEGG pathway database [19].

3. Results

3.1. Patient characteristics

At the time of recruitment, all the 8 patients were in haematological remission. 3 patients were in complete cytogenetic remission; 2 in partial and 3 in minor cytogenetic remission. All have been on IM for more than 20 months (range 21.09–120.05 months). 4 patients were switched to nilotinib. Median time to best cytogenetic response was 36.74 months (range 10.15–134.93 months) (Table 1).

Table 3b
TargetScan predicted interactions for miR-99b-5p and their putative gene targets.

TargetScan Predicted Interactions for miR-99b-5p			
	Gene Name	Gene Ensembl id	Context Score
1	*NOX4*	ENSG00000086991	−0.502
2	*ST6GALNAC4*	ENSG00000136840	−0.536
3	*PCSK9*	ENSG00000169174	−0.522
4	*KBTBD8*	ENSG00000163376	−0.578
5	*FGFR3*	ENSG00000068078	−0.444
6	*ATP11C*	ENSG00000101974	−0.498
7	*SIAE*	ENSG00000110013	−0.492
8	*ETFDH*	ENSG00000171503	−0.634
9	*RNF144B*	ENSG00000137393	−0.449
10	*RAVER2*	ENSG00000162437	−0.42
11	*SUDS3*	ENSG00000111707	−0.503
12	*EPDR1*	ENSG00000086289	−0.681
13	*VNN1*	ENSG00000112299	−0.559
14	*ZNF197*	ENSG00000186448	−0.638
15	*HS3ST3B1*	ENSG00000125430	−0.547
16	*HS3ST2*	ENSG00000122254	−0.586
17	*TTC30A*	ENSG00000197557	−0.5
18	*SMARCA5*	ENSG00000153147	−0.412
19	*WDR48*	ENSG00000114742	−0.591
20	*CTDSPL*	ENSG00000144677	−0.468
21	*HES7*	ENSG00000179111	−0.488
22	*GMPS*	ENSG00000163655	−0.561
23	*BAZ2A*	ENSG00000076108	−0.439
24	*SATB1*	ENSG00000182568	−0.446
25	*FARP2*	ENSG00000006607	−0.405
26	*THAP2*	ENSG00000173451	−0.635
27	*PSMD1*	ENSG00000173692	−0.465
28	*NR1I3*	ENSG00000143257	−0.456
29	*ZZEF1*	ENSG00000074755	−0.407
30	*AP1AR*	ENSG00000138660	−0.522
31	*TMPRSS13*	ENSG00000137747	−0.547
32	*RAP1B*	ENSG00000127314	−0.434
33	*TNFAIP8*	ENSG00000145779	−0.594
34	*PAPL*	ENSG00000183760	−0.506

3.2. Principal component analysis and hierarchical clustering

PCA showed distinct clustering of the patient cohort and normal controls (Fig. 1). Subsequent data analysis identified 54 differentially expressed miRNAs in IM-resistant CML patients compared to normal controls; 43 were downregulated (Table 2a) and 11 were upregulated (Table 2b). Subsequent hierarchical clustering (Fig. 2) showed that the IM-resistant CML patients had a distinct miRNA expression profile compared to normal controls. The top 4 most significantly down-regulated miRNAs were miR-146a-5p, miR-7849-3p, miR-99b-5p and miR-151a-5p. However, miR-7849-3p was not further analyzed as the number of absolute reads was too small to be deemed of clinical significance.

3.3. Validation by RQ-PCR

The miRNA-Seq results for the remaining top 3 miRNAs (miR-146a-5p, miR-99b-5p and miR-151a-5p) were validated using RQ-PCR (Fig. 3).

3.4. In silico target and pathway analysis

miRPAth v.3 analysis of our dataset showed that the 3 miRNAS (miR-146a-5p, miR-99b-5p and miR-151a-5p) are predicted to regulate multiple gene targets (Tables 3a–3c). When these were merged by way of gene targets using the KEGG database, *ATRIP, ATR, WDR48, RAD51C* and *FANCA* were found to be putative target genes (Table 4) that are all involved in the Fanconi Anemia (FA) pathway (p-value = 0.012) (Fig. 4).

Table 3c
TargetScan predicted interactions for miR-151a-5p and their putative gene targets.

TargetScan Predicted Interactions for miR-151a-5p			
	Gene Name	Gene Ensembl id	Context Score
1	*EFNB2*	ENSG00000125266	−0.59
2	*NTRK2*	ENSG00000148053	−0.538
3	*NBAS*	ENSG00000151779	−0.463
4	*RALGAPA1*	ENSG00000174373	−0.515
5	*GLT1D1*	ENSG00000151948	−0.514
6	*ZNF208*	ENSG00000160321	−0.532
7	*PTGDR2*	ENSG00000183134	−0.471
8	*ZNF532*	ENSG00000074657	−0.405
9	*LPPR5*	ENSG00000117598	−0.56
10	*TRMT44*	ENSG00000155275	−0.424
11	*PQLC1*	ENSG00000122490	−0.747
12	*CACNG7*	ENSG00000105605	−0.407
13	*SOX17*	ENSG00000164736	−0.754
14	*IQGAP1*	ENSG00000140575	−0.496
15	*MEST*	ENSG00000106484	−0.412
16	*APH1A*	ENSG00000117362	−0.438
17	*DDX20*	ENSG00000064703	−0.508
18	*HS3ST1*	ENSG00000002587	−0.445
19	*C2*	ENSG00000166278	−0.578
20	*B3GALT5*	ENSG00000183778	−0.521
21	*SLC7A5*	ENSG00000103257	−0.462
22	*SFT2D3*	ENSG00000173349	−0.424
23	*IGSF9B*	ENSG00000080854	−0.689
24	*TDRD3*	ENSG00000083544	−0.471
25	*SERPINF2*	ENSG00000167711	−0.423
26	*UTY*	ENSG00000183878	−0.483
27	*LYPD3*	ENSG00000124466	−0.564
28	*C9orf47*	ENSG00000186354	−0.411
29	*MED16*	ENSG00000175221	−0.402
30	*AC011366.3*	ENSG00000254106	−0.505
31	*LARP4B*	ENSG00000107929	−0.425
32	*SH3BP4*	ENSG00000130147	−0.407
33	*NDE1*	ENSG00000072864	−0.761
34	*TSC2*	ENSG00000103197	−0.542
35	*TRPM2*	ENSG00000142185	−0.437
36	*CLEC4F*	ENSG00000152672	−0.443
37	*SIGLEC12*	ENSG00000254521	−0.553
38	*HOXA3*	ENSG00000105997	−0.433
39	*RAD51C*	ENSG00000108384	−0.461
40	*PATZ1*	ENSG00000100105	−0.467
41	*SEZ6L*	ENSG00000100095	−0.545
42	*GDI1*	ENSG00000203879	−0.502
43	*PRODH*	ENSG00000100033	−0.491
44	*MTRR*	ENSG00000124275	−0.535
45	*RNF165*	ENSG00000141622	−0.512
46	*CYB5B*	ENSG00000103018	−0.484
47	*AC005609.1*	ENSG00000249034	−0.524
48	*FANCA*	ENSG00000187741	−0.562
49	*AC013269.5*	ENSG00000184761	−0.427
50	*SYMPK*	ENSG00000125755	−0.461
51	*GALP*	ENSG00000197487	−0.516
52	*DNMBP*	ENSG00000107554	−0.544
53	*SCN8A*	ENSG00000196876	−0.447
54	*N4BP1*	ENSG00000102921	−0.449
55	*RABL6*	ENSG00000196642	−1.408
56	*KIAA1429*	ENSG00000164944	−0.421
57	*MPPED1*	ENSG00000186732	−0.532
58	*CARHSP1*	ENSG00000153048	−0.459
59	*ERI1*	ENSG00000104626	−0.417
60	*ASCL5*	ENSG00000232237	−0.46

4. Discussion

In the era of IM for the treatment of CML, it is becoming increasingly clear that IM-resistance represents an emerging issue. It has been estimated that a third of all newly diagnosed CML patients will invariably fail to achieve optimal response with IM [20,21]. IM-resistance due to point mutations in the *BCR-ABL* KD was almost immediately identified in the trial phases of the drug [22] and numerous other additional KD mutations leading to amino acid substitutions have since been described. *BCR-ABL* KD mutations continue to represent the main cause of IM-resistant disease [23,24]. Many factors have been implicated in *BCR-ABL* independent resistance; such as drug bioavailability, alternative signalling pathways, gene hypermethylation and epigenetic modifications [25–27].

Table 4
Putative target genes that involved in the Fanconi Anemia pathway for the top 3 differentially expressed miRNAs by TargetScan.

miRNA	Gene Name	Gene Ensembl ID	Context Score
miR-146a-5p	ATRIP	ENSG00000164053	−0.449
	ATR	ENSG00000175054	−0.422
miR-99b-5p	*WDR48*	ENSG00000114742	−0.591
miR-151a-5p	*RAD51C*	ENSG00000108384	−0.461
	FANCA	ENSG00000187741	−0.562

The role of miRNAs has been a subject of intense research. Recently it was shown that plasma miR-215 was downregulated in the STOP-IM group compared to the control, suggesting a biological role of this miRNA in CML patients who have stopped IM and maintained undetectable minimal residual disease [28]. In a comparison between IM-responders and non-responders, Lin et al. reported 7 differentially expressed mRNAs that were predicted targets of the dysregulated miRNAs identified [29]. Other miRNAs have been implicated in TKI-resistance and were extensively reviewed [7] but no unifying pathway has been definitively identified. Interestingly, in our study, we showed that IM-resistance may be influenced by miRNAs that affect the FA pathway. MiRNA expression has been known to differ between CML cell lines and normal controls [30]. Our patients were heavily pre-treated and in haematological remission. It was encouraging to note that even so, there was a clear characteristic miRNA expression signature compared to normal controls. MiRNAs can regulate numerous target genes and therefore could modulate multiple pathways. Thus it is of significance that the 3 most differentially downregulated miRNAs in our cohort were found to interact within the FA pathway.

The FA pathway coordinates a complex mechanism that enlists elements of 3 classic DNA repair pathways, namely homologous recombination, nucleotide excision repair, and mutagenic translesion synthesis; as part of the DNA damage response (DDR) [31]. The pathway is made up of 3 core protein complexes comprising of 13 known proteins (Fig. 4). Once triggered by ATR, the core complexes migrate to DNA damage sites and ubiquitinate downstream molecules and co-localise with other proteins such as RAD51 and BRCA to initiate DNA repair. Besides its namesake Fanconi anemia; a disease characterized by bone marrow failure, developmental defects, and cancer proneness, the FA pathway has also been implicated in chemoresistance in other malignancies [32]. The full extent of the FA pathway has not yet been elucidated and many proteins involved in it also participate in other cellular mechanisms such as JAK/STAT signalling and cytokinetic regulation [33,34].

miR-146a-5p has been reported to be upregulated in newly-diagnosed CML patients and normalization of this miRNA was seen after two weeks of IM therapy [8]. The down-regulation of this miRNA in our patients suggests an additional role in conferring IM resistance. The putative gene targets of miR-146a-5p, *ATRIP* and *ATR*, encode for two mutually dependent kinases that are essential for signalling the presence of DNA damage and activating cell cycle checkpoint. *ATR* is a main upstream regulator of the FA pathway and phosphorylates *FANCD2, FANCI, FANCG* and *FANCA*, affecting a phosphorylation-ubiquitination cross-interaction to induce crosslink in DDR [35]. Aside from serving as an upstream regulator of the FA pathway, *ATR* is also a member of the phosphoinositol-3-kinase family which are major players in the anti-apoptotic PI3K/AKT pathway [36,37]. Recently, it was reported that the PI3 K/AKT pathway was activated in conjunction with IM resistance in gastrointestinal stromal tumors, another tyrosine kinase driven malignancy. As well, studies in CML have shown that

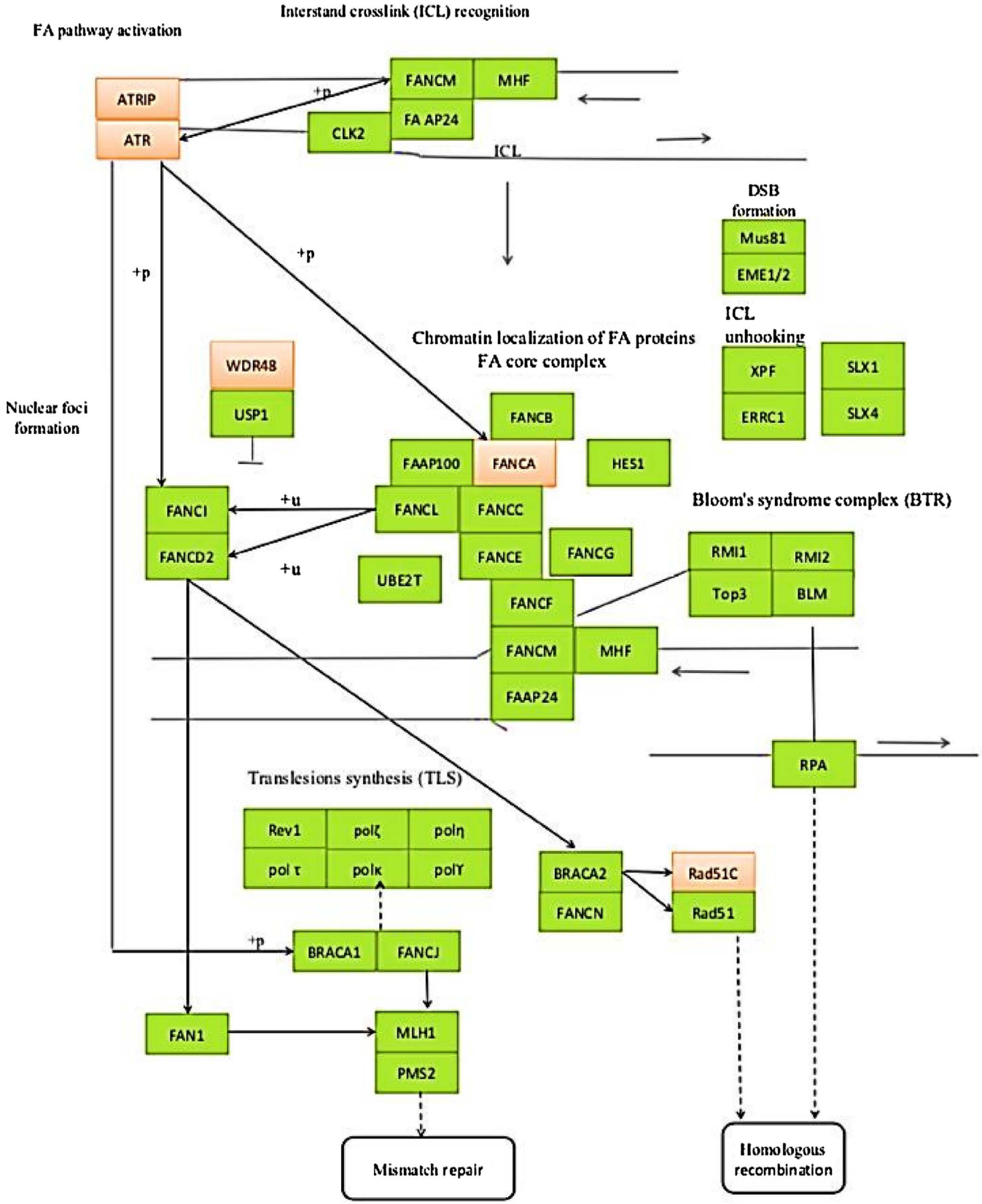

Fig. 4. Fanconi Anemia Pathway.
http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map03460&keyword=fanconi%20anemia.
(adapted from 03460 11/21/12 © Kanehisa Laboratories).

this occurs independently from *BCR-ABL* [38,39]. PI3K/AKT pathway modulation by the mTOR inhibitor everolimus for IM-resistant CML patients has entered clinical trials. (ClinicalTrials.gov Identifier: NCT00093639). Further study is needed to elucidate the spectrum of ATR-miR146a interplay in IM resistance.

RAD51C and *FANCA* are putative gene targets of miR-151a-5 which is another significantly downregulated miRNA in our patient cohort. RAD51C is a multimeric helical nucleoprotein filament that acts downstream of the FA pathway that ensures efficient DNA double-strand break repair by homologous recombination. Mutations in *RAD51* has been implicated in various malignancies [40,41]. In CML, the mechanism by which *BCR-ABL* renders resistance to genotoxicity is believed to be achieved in part via overexpression of *RAD51* [42,43]. Since *RAD51* overexpression has been reported in other non-fusion tyrosine kinase-related tumours as well as sensitivity to treatment in these tumours, it is reasonable to speculate that *RAD51* may be independent of *BCR-ABL* [44]. *FANCA*, along with *FANCB, FANC, FANCE, FANCF, FANCG, FANCL* and *FANCM,* is a part of the core complex of the FA pathway which is an ubiquitin ligase that is central to the pathway's functionality. As mentioned above, *FANCA* has been shown to be phosphorylated in the event of DNA damage and likely integral to DNA repair.

miR-99b-5p targets *WDR48* gene which enhances the USP1-mediated deubiquitination of FANCD2, thereby regulating deubiquitinating complexes within the FA pathway [45]. This process is a negative feedback mechanism that keeps the FA pathway in check [46].

Patients with CML who are resistant to IM possess a distinct miRNA signature. Our research also demonstrated for the first time that non-KD mutation related IM-resistance is influenced by miRNAs that also affect targets within the FA pathway. This provides a unique angle into the pathogenesis of IM-resistance; giving rise to potential biomarkers of TKI resistance or adjunctive targeted therapy in addition to a TKI. Another question arising is the role of second generation TKIs within this *BCR-ABL*- independent hypothesis: only 2 out of 4 of our patients who switched to nilotinib achieved partial cytogenetic response as best response (Table 1).

Our results disclosed several identical miRNAs (Table 2a–c). This can be attributed to sequencing or alignment artefacts. However, miRNA-seq enables the detection of isomiRs, an interesting and not-fully resolved feature of miRNA biology. Some isomiRs have been shown to act co-operatively or competitively with their canonical counterparts to exert meaningful biological effects [47,48]. Further studies are needed to clarify this phenomenon in IM-resistant CML.

Our study is limited by a relatively small sample size and only 2 control individuals. As well, in our clinical practice, *BCR ABL* mutation analysis was not consistently available and thus were performed at various time points after patients demonstrated clinical resistance to IM. Hence, we are unable to pinpoint the exact time of KD mutation acquisition; diminishing our ability to distinguish patients who have primary or secondary IM resistance.

KD mutation-negative IM-resistance remains a heterogeneous phenomenon stemming from multiple molecular events occurring independently. This further highlights the complexity of this unmet clinical need and its management. Further research is needed to elucidate the role of miRNA expression in modulating these genes and their interactions in promulgating an IM-resistant phenotype.

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