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ORIGINAL ARTICLE

MicroRNA (miRNA) expression profiling of peripheral blood samples in multiple myeloma patients using microarray

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Abstract

MicroRNAs (miRNAs) are mostly located at cancer-associated genomic regions or in fragile sites, suggesting their important role in the pathogenesis of human cancers. Multiple myeloma (MM) is a cancer of plasma cells, the third most common cancer of the blood after lymphoma and leukaemia. There are several published reports on miRNAs in MM, however most used bone marrow rather than peripheral blood samples. The aim of this study is to characterise miRNA expression in normal and MM patients using peripheral blood samples as it is less invasive and is readily available from patients. Blood samples from 35 MM patients were analysed using the microarray method. We identified up-regulation of 36 miRNAs (57%) and down-regulation of 27 miRNAs (43%). We also identified the CCND2, HMGA2 and IGF1R genes were among the highly predictive target genes (P_{CT}> 0.80) for most of the deregulated miRNAs. These genes are known to play important roles in MM as well as other cancers. Five miRNAs (let-7c, miR-16, miR- 449, miR-181a and miR-181b) were found to exhibit similar expression patterns ($p < 0.05$) in peripheral blood when compared to data obtained by using bone marrow aspirates from MM patients in other studies. In conclusion, our study has demonstrated that miRNAs are also present and differentially expressed in the peripheral blood of MM patients compared to controls and may potentially serve as candidate tumour biomarkers in MM. In particular, let-7c and miR-16 have been shown to be significantly expressed in the bone marrow.

Keywords: MicroRNA, miRNA expression, biomarker, multiple myeloma

INTRODUCTION

Multiple myeloma (MM), or cancer of plasma cells, accounts for approximately 10% of hematological malignancies.[1] MM is characterized by its migration and localisation to the bone marrow from where cells disseminate and facilitate formation of bone lesions[1]. MM is indicated by clinical features such as anaemia, bone lesions, hypercalcaemia and immunodeficiency. Despite all available therapies, MM is generally considered incurable with a median survival between two to three years.[2] The International Myeloma Foundation reported that MM affects approximately 20,000 individuals in the United States. The incidence among Asians is ~0.5-1/100,000, whereas the incidence among African-American is ~10-12/100,000 men.[3] In Malaysia, clinical samples of MM patients from all hospitals under the Ministry of Health are referred to the Molecular Diagnostics & Protein Unit (UMDP), Institute for Medical Research (IMR), for testing, for both screening and confirmation. IMR has served as a referral centre for MM since 1982. During a 10-year period (1999-2009), of the 2000-2200 samples received by IMR, about 130-230 of new cases were diagnosed and 250-300 follow-up cases were tested annually.[4]

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Advances in gene expression studies have revealed the existence of small non-coding RNA molecules called microRNAs (miRNAs). These miRNAs are thought to regulate the expression of thousands of human genes in a negative manner.[5] This has been a great breakthrough as for many years DNA and protein were believed to be the key players in gene expression while RNA was merely a messenger that transfers information between the DNA and protein. MiRNAs are small noncoding RNAs of 18-25 nucleotides in length that control gene expression by targeting mRNAs and triggering either translational repression or mRNA degradation via the RNA-interference pathway[5]. Nearly all genes contain short sequences that match the core portions sequences of miRNAs. miRNA can bind to the short binding sequence on a targeted mRNA and suppresses protein production. According to bioinformatics analysis, more than 60% of protein coding genes may be targeted by miRNAs.[6]

miRNAs have been shown to play a fundamental role in diverse physiological and pathological processes that include cell development, cell differentiation, apoptosis, proliferation, stress response and fat metabolism through the regulation of gene expression.[7,8] Investigations by Calin *et al*. on chronic lymphocytic leukaemia (CLL) discovered that two microRNA genes, mir-15 and mir-16, were found to be deleted or down-regulated in 68% of CLL patients.[9] CLL is the most common form of adult leukaemia, caused by deletion of 13q14 in more than 50% of cases. Until the discovery of miRNAs, none of the known genes located in this deleted region have been shown to lead to CLL.[9] Furthermore, they also found that point mutations of the miRNA genes were frequent in CLL. One study involving lung cancer found that a decreased expression of let-7 led to increased expression of the Ras oncogene. This finding correlated with poor prognosis and may contribute to tumorigenesis.[10]

Mostly, miRNAs expression profiling has been associated with solid cancerous tumours and hematologic malignancies, and yet little is known about miRNA expression in MM. The earliest study on MM by Pichiorri *et al*. described a comprehensive global miRNA expression profiling of MM, monoclonal gammopathies of undetermined significance (MGUS) and normal plasma cells.[11] They discovered distinctive miRNA signatures with known oncogenic functions (i.e. miR-21, miR-106b~25 cluster, miR-181a and b) in the malignant transformation of plasma cells in MM and MGUS. In 2009, Roccaro *et al.* validated the functional role of miR-15a and miR-16 in regulating tumour proliferation of MM cells *in vitro* and *in vivo*.[12] Further on in 2010, Gutierrez et al. investigated the association between miRNAs expression profiles and their respective target genes.[13] They found that under-expression of miR-196b, miR-135b, miR-320, miR-20a, miR-18b, miR-19a and miR-15a in MM caused an over-expression of CCND2, a predicted target gene. They were the first to report that miRNAs expression profiling is correlated with genetic abnormalities and that the inverse correlation of miRNA-mRNA interactions could be helpful in finding genes that can be regulated by specific miRNAs in the pathogenesis of MM. These evidences indicate the contribution of miRNAs in the pathogenesis of MM as well as its potential use as a molecular biomarker.

In order to use a biomarker for diagnostics, the sample source must be as easily to obtain as possible. Although MM is a disease of plasma cells, abnormalities of both B and T lymphocytes have been detected in peripheral blood.[14] It is known that the blood of cancer patients contains fragments of RNA released from the tumour and the concentration of the RNA fragments is higher in the blood of cancer patients than in the blood of healthy individuals.[15] A cohort study on MM patients by Keats *et al*. described that IgH-MMSET transcript hybrids were detectable in the peripheral blood of 80% MM patients with translocation t(4;14)+ which was previously identified as positive in a bone-marrow screening test.[16] Collado *et al.* used the gene expression profiles of circulating plasma RNA to demonstrate that several genes were significantly increased in colorectal cancer patients compared to healthy individuals, and the RNA levels returned to normal after surgical removal of the tumour.[17] Improved approach for the blood-based detection of cancer has been established by Mitchell et al. whereby they have proven that miRNAs are present in human plasma in a very stable form, and are protected from endogenous RNAse activity.[18] In this present study, we made use of the easily and readily available peripheral blood to investigate the circulating miRNA as a blood-based marker for MM detection.

MATERIALS AND METHODS

Multiple myeloma and normal samples
A total of 35 samples from multiple myeloma patients from the Haematology Department, Ampang Hospital, Kuala Lumpur, were used in this study. Peripheral blood samples were collected from 14 new cases and 21 follow-up cases upon their informed consent. The patients, 23 males and 12 females, were aged between 40 to 75 years. Blood from 7 normal individuals were obtained after a written consent to serve as controls. These individuals were confirmed negative for multiple myeloma by serum protein electrophoresis test. This research was approved by the Medical Research & Ethics Committee (MREC), Ministry of Health, Malaysia.

Patients inclusion parameter
All patients were confirmed to have MM based on various clinical presentations and biochemical findings and the presence of M-protein in serum and urine. All patients showed the presence of M-protein, a very useful cancer marker secreted by myeloma cells into the blood and urine. M-protein has been detected in more than 93% of MM patients in general.[1]

Sample processing and total RNA preparation
Blood collected in EDTA tube was transferred to a depletion filter to separate leukocytes according to a LeukoLOCK total RNA isolation system protocol (Ambion, Austin, TX). Subsequently, total RNA was extracted from the cells captured on the filter by using a TRI reagent kit and silica filters (Ambion, Austin, TX). The integrity of the RNA and the presence of miRNA were assessed by micro-capillary electrophoresis using an RNA 6000 kit and Small RNA kit (Agilent Technologies, Inc., Santa Clara CA) respectively. The RNA concentration was quantified by a Nanodrop spectrometer.

miRNA microarray analysis
Microarray analysis was performed using the Agilent platform. Briefly, 100 ng of total RNA was de-phosphorylated with calf intestine alkaline phosphatase and incubated at 37°C for 30 minutes before addition of DMSO. After that, the sample was hybridised to the miRNA 15K array (Agilent Technologies, Inc., Santa Clara CA) containing 821 miRNA probes, and the array was later scanned and analysed according to Agilent standard protocols.

Sample quality standard
In this study, high-quality miRNA expression data (>92%) were obtained on all samples. The reproducibility and reliability of the microarray data were evaluated by the QC metrics report generated by the feature extraction software (Agilent Technologies, Inc., Santa Clara CA).

Bioinformatics
(i) *Analysis of differential expression of miRNAs.*
Data analysis was carried out using the GeneSpring GX 10 Expression Analysis software (Agilent Technologies, Inc., Santa Clara CA). To identify miRNAs with unique expression profiles in newly diagnosed MM and follow-up MM, we first filtered miRNAs which were differentially expressed (fold change 1.2x or higher). These miRNAs were then selected for further statistical analysis. Since the miRNAs expression data were not normally distributed, a non-parametric test was used. A one-way ANOVA unequal variance (Welch) with $p<0.05$ for significance was applied to 89 miRNAs that passed the filtering of fold-change >1.2.

Statistical analysis of the expression levels of the filtered 150 miRNAs (with fold-change >1.2) in the newly diagnosed MM cases versus healthy subjects was carried out using t-Test, unpaired unequal variance (Welch) with $p<0.05$ for significance. A similar approach was applied to follow-up MMs (98 miRNAS with fold-change >1.2). Finally, a Venn diagram incorporating the 2 filtered miRNAs sets (newly diagnosed MM and follow-up MM) was used to identify miRNAs unique to each group.

(ii) *Interrogation of the Human MicroRNA Disease Database (HMDD)*[19]
We made comparison on our expression data with HMDD, a useful database to help in understanding the associations between microRNAs and human diseases. It is also a new way to identify novel disease-associated microRNAs. The HMDD can be accessed via http://202.38.126.151/hmdd/mirna/md/.

(iii) *Prediction of the target genes for miRNAs*[6]
To predict target genes for a particular miRNA, all miRNAs unique to each group was subjected to TargetScan analysis (http://www.targetscan.org). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites at the 3' UTR of each gene transcript that match the seed region of

the interrogating miRNA. In this study, we used P_{CT} to rank the probability of conserved targeting. P_{CT} is the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance or any other reason not pertinent to miRNA targeting. From the 63 miRNAs predicted to be significantly expressed ($p < 0.05$, fold change > 1.2), we have chosen 10 miRNAs with a similar regulation reported in this as well as in other studies.

(iv) *Validation of the miRNA profiles by qRT-PCR*
To validate our microarray data, quantitative real-time PCR (qRT-PCR) analysis was performed on 10 miRNAs: miR-24, miR-98, miR-16, let-7b, let-7c, let-7i, miR-106a, miR-100, miR-99a and miR-93. These miRNAs were chosen based on the highly significant expression obtained in this study and that they are also commonly expressed in many cancer types. Healthy controls (n=5), new MM cases (n=10) and those on follow-up (n=10) were also randomly chosen. Taqman miRNA probes and primers were used to detect and quantify the mature miRNAs. The amplification was carried out using the RT product, Taqman Universal PCR master mix and primer, and probe mix of the microRNA assay protocol (Applied Biosystems, Foster City, CA). The reactions were run in triplicates for 40 cycles on an ABI 7300 Sequence Detection system. The relative quantification $2^{-\Delta\Delta CT}$ method was used to determine changes in expression levels of MM patients relative to the healthy controls. Normalization was performed with the reference miRNA, RNU6B.

RESULTS AND DISCUSSION

MiRNAs profiles in MM patients
In this study, we determined the miRNA expression profiles in the peripheral blood of 14 newly diagnosed MMs and 21 follow-up MM cases using microarray with 821 miRNA probes (799 human miRNAs and 22 viral miRNAs). Initial analysis of the miRNAs profiling showed that some miRNAs were differentially expressed in MM patients (newly diagnosed plus follow-up patients, n=35) and these mRNAs can be distinguished from normal individuals and reference RNAs. We identified that only 63 miRNAs were significantly expressed ($p < 0.05$, > 1.2 fold change) and out of these, 36 miRNAs (57%) were found to be up-regulated whereas 27 miRNAs (43%) were down-regulated (Figure 1). Our results were essentially in agreement with Pichiorri et al. who demonstrated even dysregulation (37 up- and 37 down-regulated) of miRNAs in MM subjects versus healthy plasma cells.[11]

Subsequently, we used an unsupervised hierarchical clustering algorithm based on the average-linkage method to determine whether blood from new MM cases and follow-up MM cases could be distinguished from blood of normal inviduals according to the grouping of their expression profiles. Neither new MM cases nor follow-up MM cases could be identified as a distinct cluster (Figure 1). A *post-hoc* and Tukey HSD analysis was then carried out to determine which miRNAs were exclusively expressed in new and/or follow-up MM cases. We found that 85 and 90 miRNAs were expressed in new MM and follow-up MM, respectively. As illustrated in a Venn diagram (Figure 2), 4 out of 85 miRNAs have been identified to be exclusively expressed only in new MM and 10 miRNAs were exclusively expressed in follow-up MM. The data is summarized in Table 1. We identified that miR-16 was significantly down-regulated only in new MM but not in follow-up MM, suggesting a possible role in myeloma cell growth and proliferation.

The microarray analysis also showed that 85 miRNAs were expressed in both new and follow-up MM cases suggesting there were no distinct differences in the expression profiles between these two conditions. This could be explained by the fact that both conditions have undergone genetic abnormalities, a hallmark of MM.[20] It is also probable that both conditions share similar genetic pathways through the regulation of specific gene expression. Several common miRNAs expressed in both conditions are shown in Table 2.

Other reason that may have contributed is the heterogeneity of the peripheral blood cell population such as neutrophils, T cells, B cells, NK cells. This may cause a decrease in sensitivity for specific molecular profiling detection in both conditions. However, we have taken several measures for improving the detection sensitivity and reducing noice in peripheral blood cells by isolating the RNA from the leucocytes,[21,22] using the highly efficient direct-labelling method and novel microarray probe designed for miRNA profiling assay[23] and data analysis using quantile normalisation to remove systematic differences between samples in heterogenous tissue.[24]

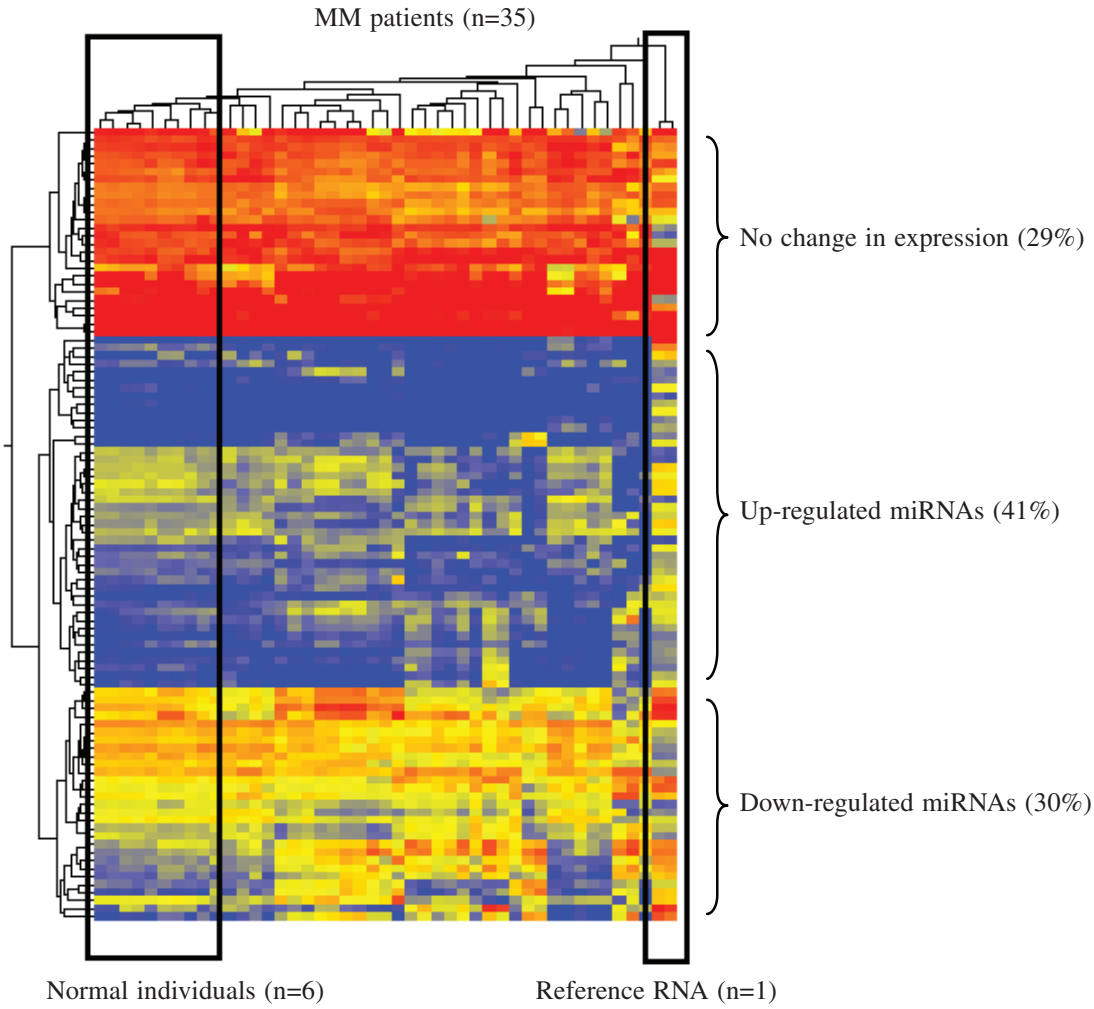

FIG. 1: Hierarchical clustering of 35 MM patients, 6 normal individuals and 1 reference RNA (columns) versus 89 miRNAs (rows). All 42 samples were clustered according to their similarity in expression pattern over all the samples ($p < 0.05$). Red indicates no changes in expression, blue indicates up-regulation and yellow indicates down-regulation in miRNA expression. (Filtered data: 89 out of 821 miRNA probes with fold-change >1.2)

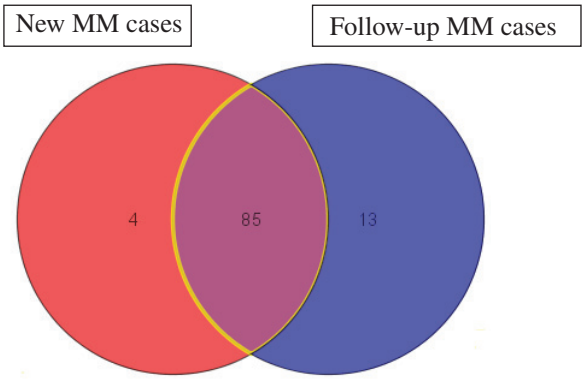

FIG. 2: Common miRNAs (n=85) between new and follow-up MM cases. Four miRNAs are exclusively identified in the new MM and 13 miRNAs were in the follow-up MM cases.

TABLE 1: MiRNAs exclusively expressed in the new and follow-up MM cases ($p < 0.05$, fold change >1.2)

miRNAs	Corrected p-value	p-value	Fold change ([MM] vs [controls])	Regulation
New MM cases				
hsa-miR-494	0.045	0.032	1.2	nc
hsa-miR-130a	0.049	0.036	2.1	down
hsa-let-7c	0.040	0.026	1.2	down
hsa-let-7i	0.014	0.007	1.4	down
Follow-up MM cases				
hsa-miR-148a	0.021	0.013	1.0	nc
hsa-miR-1225-5p	0.022	0.014	1.4	up
hsa-miR-423-5p	0.001	0.000	2.0	up
hsa-miR-484	0.019	0.012	1.5	up
hsa-miR-99a	0.014	0.008	2.1	up
hsa-miR-106a	0.030	0.021	1.2	up
hsa-let-7b	0.001	0.000	1.4	up
hsa-miR-224	0.017	0.010	2.0	up
hsa-miR-638	0.015	0.008	2.0	up

nc, no change. Corrected p-value: p-value*n (number of genes in test) < 0.05
(If the corrected p-value is still below the error-rate, the gene will be significant)

Similarity of miRNAs expression pattern observed in the blood and also in other studies

Five miRNAs i.e. let-7c, miR-16, miR-449, miR-181a and miR-181b, showed similar expression patterns as in other MM studies using bone marrow aspirates (Table 3). Pichiorri *et al.* reported that miR-181a and miR-181b were associated with neoplastic transformation and development of MM and confirmed the role of both miRNAs as onco-miRNAs by *in vivo* studies.[11] A report by Roccaro *et al.* demonstrated that down-regulation of miR-15a and miR-16 in MM patients regulated the proliferation and growth of myeloma cells *in vitro* and *in vivo*.[12]

We further used our data to interrogate the Human miRNA and Disease Database (HMDD) and found 65% of deregulated miRNAs identified

TABLE 2: Selected miRNAs that were expressed in both the new and follow-up MM cases ($p < 0.05$, fold change > 1.2)

miRNAs	New MM cases		Follow-up MM cases	
	Fold-change	Regulation	Fold-change	Regulation
hsa-miR-324-3p	1.291	up	1.4	up
hsa-miR-103	1.52	up	1.0	nc
hsa-miR-155	1.208	up	1.1	nc
hsa-miR-125b	1.763	down	1.5	up
hsa-miR-98	2.413	down	1.3	down
hsa-miR-15a	1.296	down	1.4	up
hsa-miR-16	2.084	down	1.1	nc
hsa-miR-93	1.007	nc	1.3	up
hsa-miR-24	1.192	nc	1.7	down
hsa-miR-22	1.149	nc	2.0	up

The ten miRNAs shown were selected from the 85 miRNAs that were common between the new and follow-up cases. nc, no change.

TABLE 3. Similarity of miRNAs regulation patterns expressed in blood in this study with bone marrow aspirate from other studies

No	miRNA	Regulation	Cancer type	Reference
1	Let-7c	down	MM	Gutierrez et al. 2010 [13]
2	miR-16	down	MM	Roccaro et al. 2009 [12]
3	miR-449	up	MM	Gutierrez et al.2010 [13]
4	miR-181a	up	MM, MGUS	Roccaro et al. 2009 [12] Pichiorri et al. 2008 [11]
5	miR-181b	up	MM, MGUS	Roccaro et al. 2009 [12] Pichiorri et al. 2008 [11]

MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

in this study have been annotated as cancer-associated miRNAs (data not shown). For instance, miR-93, which was up-regulated in our study, has also been shown to be up-regulated in cancers of ovary, stomach, prostate, melanoma and glioma. Down-regulation of miR-16 in our study is also in agreement with regulation in B-CLL, pituitary and prostate cancer. This analysis suggests that deregulated miRNAs expressed in the blood of MM patients also have significant roles as reported in various cancer types as in Table 4.

Our study also showed that MM patients have differentially expressed miRNAs circulating in their peripheral blood (Table 2) and that some of them, such as miR-16, may be potential cancer markers since their expression is detectable in the blood. Cancer-associated miRNAs are detected in the blood circulation due to lysis of dead cancer cells, or were released by cancer cells into the environment,[25] hence their potential as biomarkers for cancer.

Validation of the miRNA profiles by qRT-PC

We confirmed down-regulation of miR-24, miR-98, miR-16, let-7c and let-7i in MM patients with respects to healthy individuals. Consistent with the microarray data, the let-7b and miR-106a showed up-regulation expression in MM patients when compared to healthy indviduals. Validation data suggest that these miRNAs are MM-specific.

Target genes for Let-7b, Let-7c, Let-7i, miR-16, miR-106a, miR-24 and miR-98

We next analysed the predicted gene targets for each up- or down-regulated miRNA using TargetScan. We found that all miRNAs are likely targeting oncogenes or tumour suppressor genes (Table 5). Predicted target genes of the up-regulated miRNAs in MM patients included CCND2, CCND1 and FGFR1. On the other hand, predicted target genes for the down-regulated miRNAs in MM included HMGA2, IGF1R and BCL2L11. These genes are known to play important roles in MM as well as in other cancers. The 3'UTR region of CCND2 gene has the most (7) binding sites for the 5 miRNAs analysed and these putative binding sites are targeted by the miRNAs to suppress translation of the CCND2 protein in MM patients (Figure 5). Bartel et al. described that translational repression requires one or several miRNAs, identical or different, to bind to the 3'UTR of the particular target mRNA.[5] Decreased miR-16 expression has been shown to inhibit CCND1, CCND2 and

TABLE 4. Similarity of miRNAs regulation patterns expressed in blood of MM patients with other type of cancers

No	miRNA	Regulation	Cancer type	Reference
1	miR-16	down	CLL	Calin et al. 2002 [9] Cimmino et. al. 2005 [27] Linsey et. al. 2007 [28]
2	Let-7 (a,-b,-c,-d)	down	Lung & breast	Takamizawa et al. 2004 [10] Jiang et. al. 2005 [29]

CLL, chronic lymphocytic leukaemia

TABLE 5. Prediction of mRNA-miRNA interaction

No	miRNA[a]	Target mRNA	No of binding site	P_{CT} [b]	mRNA function (TS/OG)
1	Let-7b/ Let-7c/ Let-7i/ miR-98	CCND2	3	0.93	OG
		CCND1	2	0.88	OG
		HMGA2	5	0.90	OG
		IGF1R	3	0.98	OG
2	miR-16	CCND2	3	0.89	OG
		CCND1	2	0.88	OG
		FGFR1	2	0.75	OG
		CCNE1	2	0.80	OG
3	miR-106a	CCND1	1	0.87	OG
		CCND2	1	0.85	OG
		BCL2L11	1	0.93	OG
4	miR-24	BCL2L11	2	0.87	OG
		IGFBP5	1	0.75	OG
		NA1F1	1	0.83	TS

[a]All miRNAs exhibited down-regulation except up-regulation for miR-16 and Let-7b. [b]A prediction score of $P_{CT} > 0.75$ was used to predict sites with higher probability of preferential conservation across species. TS, tumour suppressor; OG, oncogene

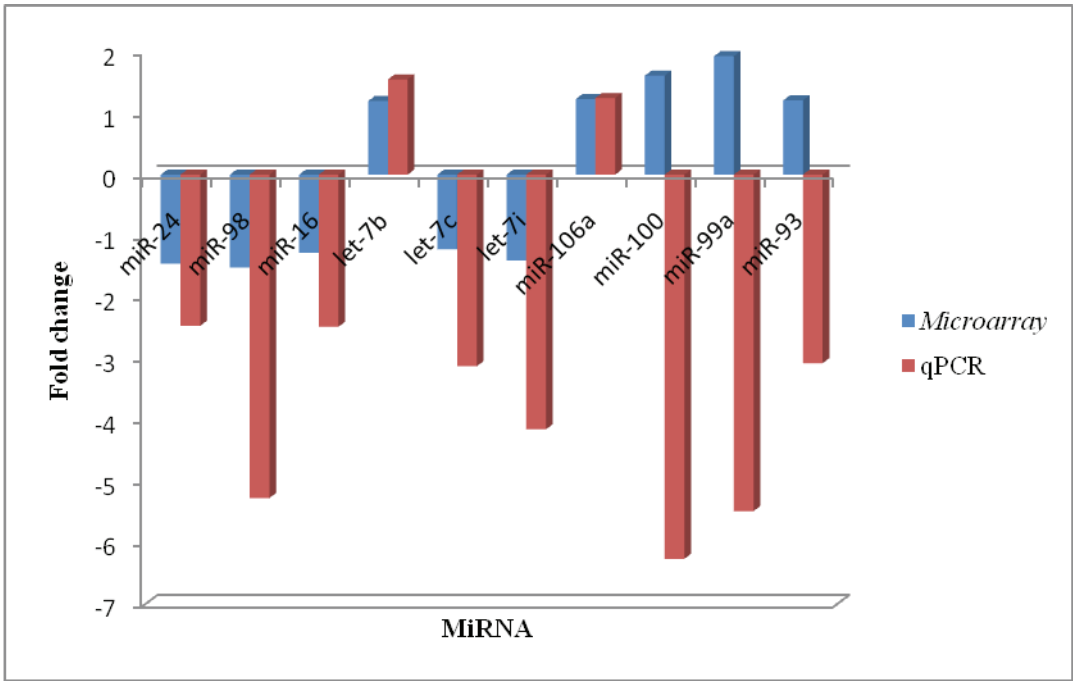

FIG. 3 Validation of the miRNA profiles by qRT-PCR. Expression level of 10 miRNAs; miR-24, miR-98, miR-16, let-7b, let-7c, let-7i, miR-106a, miR-100, miR-99a and miR-93 in MM patients (n=24) were compared to normal individuals (n=5). Result showed 70% of the expression pattern in qPCR (red) was similar to those observed in microarray (blue). The qPCR data obtained by using the $2^{-\Delta\Delta CT}$ method normalised with RNU6B as a reference.

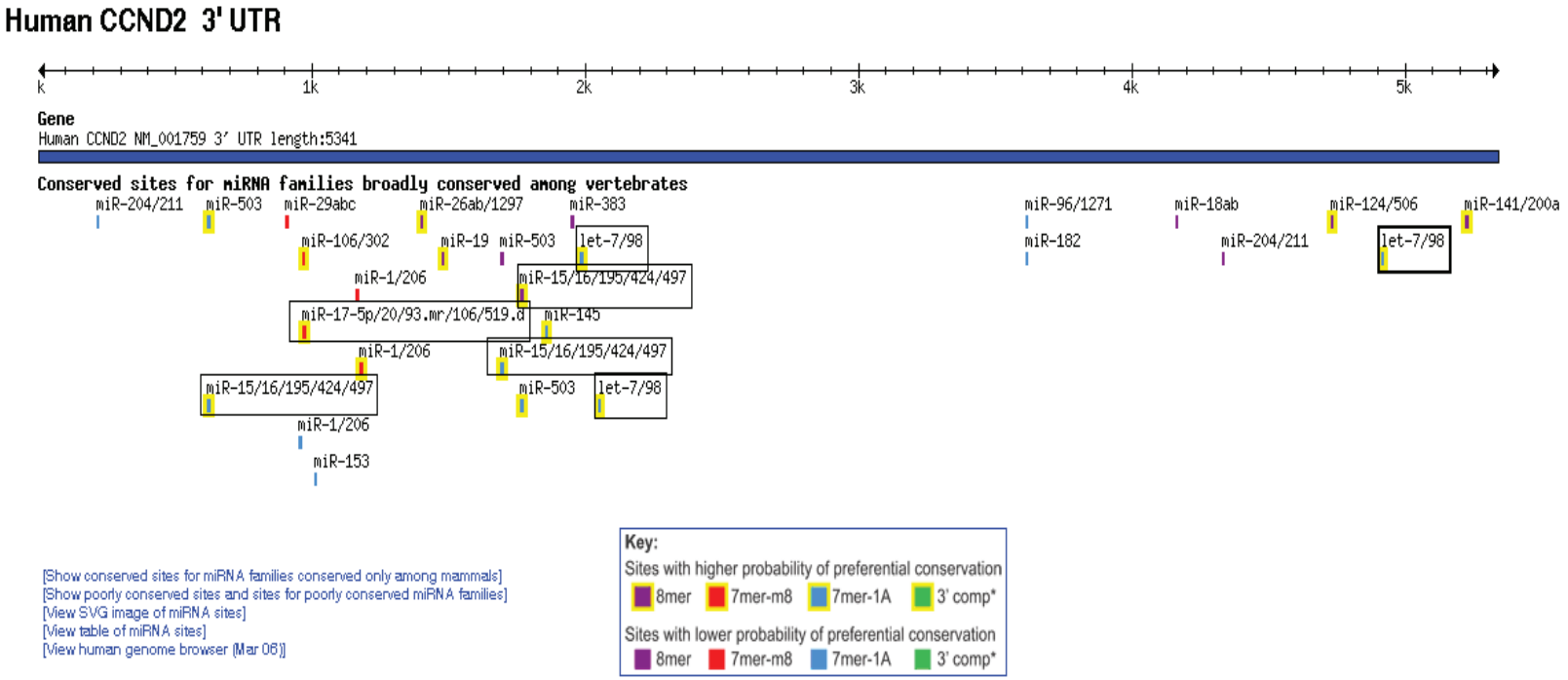

FIG. 4 The 3'UTR region of human CCND2 mRNA with a conserved binding sites for let-7b, let-7c, let-7i, miR-98, miR-16 and miR-106a. The let7/98 group and miR-16 include 3 conserved binding sites with P_{CT} between 0.81-0.93, whereas miR-93/miR-106a include 1 binding site, P_{CT} =0.89. The binding site location is boxed. Prediction was made by interrogating the database *TargetScan Human 5.1: Prediction of microRNA targets (2009).*

CDC25A expression in MM cells[12], supporting our prediction that miR-16 has a significant role in regulating cell cycle by inhibiting CCND1 and CCND2. Dysregulation of CYCLIN D gene has been proposed as an early and unifying oncogenic event in MM[26], with CCND2 being expressed in 45% of MM cases. Therefore, down-regulation of let-7b, let-7c, let-7i, miR-16 and miR-98 found in our study are predicted to contribute to dysregulation of the CCND2 gene. The other targeted genes, HMGA2 and IGFR1, are also known to be involved in cell cycle and play important roles in the control of MM cell biology, respectively. These miRNAs may also function as tumour supressors by targeting and suppressing mainly oncogenes as tabulated in Table 5.

Conclusions

In conclusion, our study has demonstrated that miRNAs are also present and differentially expressed in the peripheral blood of MM patients compared to controls and may potentially serve as candidate tumour biomarkers in MM. In particular, let-7c and miR-16 have been shown to be significantly expressed in the bone marrow.

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