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ORIGINAL ARTICLE

Downregulated plasma miR-92a levels have clinical impact on multiple myeloma and related disorders

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Recent studies have demonstrated that one-third of known microRNAs (miRNAs) are stably detectable in plasma. Therefore, we assessed plasma miRNAs to investigate the dynamics of oncomir 17-92a, which is highly expressed in multiple myeloma (MM) patients. The plasma miR-92a level in symptomatic MM patients was significantly downregulated compared with normal subjects ($P < 0.0001$), regardless of immunoglobulin subtypes or disease stage at diagnosis. In contrast, miR-92a levels in peripheral blood CD8$^+$ or CD4$^+$ cells from MM patients were lower than those of normal subjects, and the miR-92a levels of the cells tended to correlate with plasma miR-92a levels. The plasma miR-92a level in the complete remission group became normalized, whereas the partial response (PR) and very good PR groups did not reach the normal range. In smoldering MM, the plasma miR-92a level did not show a significant difference compared with normal subjects. Our findings suggest that measurement of the plasma miR-92a level in MM patients could be useful for initiation of chemotherapy and monitoring disease status, and the level may represent, in part, the T-cell immunity status of these patients.

INTRODUCTION

MicroRNAs (miRNAs) consist of approximately 18–22-nucleotide non-coding RNA molecules that regulate posttranscriptional gene expression by degradation or repression of mRNA molecules.[1-3] Individual miRNAs can target multiple mRNAs and control transcription in approximately one-third of human genes. Recent studies have shown that these miRNAs are closely involved in cell differentiation, proliferation, apoptosis or oncogenesis.[1-3]

In various human cancer, there is evidence of the alteration of tumor tissue-specific miRNA.[4-6] A 2008 study demonstrated that miRNAs stably exist in serum and plasma,[7,8] and a recent advance revealed the presence of circulating miRNAs within lipoprotein (known as microvesicles), which may help to protect the miRNAs from RNase-dependent degradation.[9,10] Moreover, some extracellular circulating miRNAs in blood plasma are independent of exosomes and are bound to Ago2 protein, resulting in strong nuclease/proteinase resistance,[11] thus indicating a possible role of circulating miRNAs in healthy subjects and disease condition.[7,8] The biological relevance of circulating miRNAs remains unclear, although they may have an important role in cancer metastasis or neo-angiogenesis. Therefore, circulating miRNAs are thought to be possible diagnostic or prognostic biomarkers of human diseases.[12-14]

It is well-known that multiple myeloma (MM) cells have a high expression level of the miR-17-92a cluster,[15] whereas plasma miR-92a levels in acute lymphoid leukemia or non-Hodgkin's lymphoma are extremely downregulated.[16-18] Downregulation of miRNAs, let-7a and miR-16 in myelodysplastic syndromes also has been reported, and their levels were significantly associated with progression-free survival and overall survival, suggesting that certain miRNAs in plasma can serve as noninvasive biomarker in

hematologic malignancies.[19] To gain more insight into the clinical relevance of plasma miR-92a expression, we evaluated plasma miR-92a levels in MM patients at various phases and in patients with related disorders. In addition, we examined cellular miR-92a levels in circulating lymphocytes obtained from untreated MM patients and compared them with the plasma miR-92a level to ascertain the possible association between immunological condition and plasma miR-92a expression.

MATERIALS AND METHODS

Patients and samples

We evaluated peripheral blood obtained from 168 patients with monoclonal gammopathies: 138 with symptomatic MM, 8 with smoldering MM (SMM) and 22 with monoclonal gammopathy of undetermined significance (MGUS). The diagnosis of monoclonal gammopathy was based on the definition of the International Myeloma Working Group using the level of serum M-protein, proportion of plasma cells in bone marrow and the presence of end-organ damage.[20] At the time of plasma collection, the disease status of the 138 patients with symptomatic MM was as follows (Table 1): 62 newly diagnosed, 8 complete remission (CR), 11 very good partial response (VGPR), 15 partial response (PR), 14 stable disease and 28 progressive disease. None of the 62 newly diagnosed MM patients had del(13q) anomaly, where the miR-17-92a located, according to the standard cytogenetic technique. Of the eight SMM patients, two had received cytotoxic therapy because of an increase in M-protein ($\geqslant 5$ g/dl). No MGUS patients had received chemotherapy before plasma collection. We analyzed plasma from 113 normal individuals and isolated lymphocytes from 21 healthy volunteers as the control. These samples were handled similarly to those obtained from MM patients.

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Table 1. Plasma miR-92a/miR-638 expression levels in monoclonal gammopathies

	Number of subjects	Median	Mean	Standard error	Minimum	Maximam	95% CI
Normal	113	1.244	1.388	0.06993	0.2154	3.58	1.249–1.527
Symptomatic MM at diagnosis	62	0.05202	0.1008	0.01899	0.000045	0.8586	0.06285–0.1388
Myeloma subtype							
Ig G	26	0.06107	0.121	0.03378	0.000045	0.8586	0.05146–0.1906
Ig A	12	0.1508	0.05435	0.1901	0.006992	3.127	0.3020–1.089
Ig D	3	0.1667	0.2353	0.1423	0.0304	0.5087	−0.3769–0.8475
Bence–Jones protein	14	0.03866	0.0973	0.04252	0.004289	0.6177	0.005448–0.1892
Non-secretory myeloma	4	0.06367	0.07089	0.02974	0.01013	0.1461	−0.02376–0.1655
Plasma cell leukemia	3	0.02628	0.03341	0.01985	0.003151	0.07081	−0.05201–0.1188
Disease status of MM							
Newly diagnosed	62	0.05202	0.1008	0.01899	0.000045	0.8586	0.06285–0.1388
CR	8	1.494	1.602	0.354	0.2285	3.643	0.7653–2.439
VGPR	11	0.2176	0.3591	0.09528	0.04138	1.028	0.1468–0.5714
PR	15	0.8179	0.7967	0.2018	0.1216	1.979	0.5409–1.052
Stable disease	14	0.3404	0.4251	0.2634	0.003508	0.9965	0.2479–0.6022
Progressive disease	28	0.06335	0.2018	0.04978	0.003319	0.8586	0.09962–0.3039
SMM	8	0.7943	1.188	0.4791	0.09087	5.242	0.3002–1.348
MGUS	22	0.4055	0.6403	0.1309	0.007652	2.428	0.3681–0.9124

Abbreviations: CI, confidence interval; CR, complete remission; MGUS, monoclonal gammopathy of undermined significance; MM, multiple myeloma; PR, partial response; SMM, smoldering MM; VGPR, very good partial response. Disease status of multiple myeloma was categorized by International Myeloma Workshop Consensus criteria.[20]

This study was approved by the institutional review board of Tokyo Medical University (no. 930, approved 24 June 2008). Written informed consent was obtained from all the participants prior to the collection of specimens according to the Declaration of Helsinki.

TaqMan low-density array screening

Total RNA was isolated with the mirVana PARIS kit (Ambion, Austin, TX, USA). Plasma samples from five patients or five healthy individuals were mixed evenly, and 500 µl of mixed plasma was diluted with 500 µl of binding solution. After a 5-min incubation, 1 µl of 1 nM ath-miR-159 (Hokkaido System Science, Hokkaido, Japan) was added to each aliquot, followed by vortexing for 30 s and incubation on ice for 10 min. Subsequent phenol extraction and filter cartridge work was performed according to the manufacturer's instructions. In all, 3 µl of RNA solution from the 50-µl elute was used as an input in each reverse transcription (RT) reaction. The RT reaction and pre-amplification step were set up according to the manufacturer's recommendations. miRNAs were reverse transcribed with the Megaplex Primer Pools (Human Pools A v2.1; Applied Biosystems, Foster City, CA, USA). RT reaction products from the plasma sample were further amplified with Megaplex PreAmp Primers (Primers A v2.1). The expression profile of the miRNAs was determined with the Human TaqMan miRNA Arrays A (Applied Biosystems). Quantitative RT-PCR was performed on an Applied Biosystems 7900HT thermocycler according to the manufacturer's recommended program. With the use of SDS2.2 software and DataAssist (Applied Biosystems), the expression of plasma miRNAs was calculated based on their Ct values normalized by those of ath-miR-159, which was spiked in each plasma sample.

miR-92a quantitative RT-PCR

Total RNA in cells was isolated with an miRNeasy Mini Kit (Qiagen, Germantown, MD, USA), and RNA in plasma was extracted as reported previously.[17,18] miRNAs were quantified with TaqMan MicroRNA Assays (Applied Biosystems) with modification and miRNA-specific stem-loop primers (has-miR-92a, 000431; has-miR-638, 001582; Applied Biosystems), as we reported previously.[17,18] The plasma miR-92a expression was normalized to miR-638 expression, and the cellular miR-92a expression levels were normalized to RNU6B (001093; Applied Biosystems). We have never detected U6B in a plasma sample, although U6B is commonly used as an internal standard for miRNA expression analysis in cells. To normalize

the expression level of miR-92a in plasma, we compared plasma miR-92a expression using an external standard, cel-miR-39 (Hokkaido System Science), as well as an internal standard, miR-638, which is stably detected in all samples. The results using miR-638 are compatible with those using cel-miR-39 (data not shown). We therefore used miR-638 as a reference in each plasma sample, as previously reported.[17,18]

Lymphocyte separation

The CD4$^+$ or CD8$^+$ T-cell fractions were separated with an isolation kit for humans (Miltenyi Biotec, Bergisch Gladbach, Germany) and AutoMACS Pro Separator (Miltenyi Biotec), according to the supplier's instruction, and stored at −80 °C until use.

Statistical analysis

GraphPad 5.0 software (GraphPad Software Inc, San Diego, CA, USA) was used for statistical analysis. The Mann–Whitney test was used to determine statistical significance between two groups and one-way ANOVA for three or more groups. We also used the Chi-square test and Student's *t*-test, when appropriate. *P*-values <0.05 were considered to indicate statistically significant differences.

RESULTS

Identification of differentially expressed plasma miRNAs in MM patients and healthy volunteers

Plasma miRNA expression was initially screened using the TaqMan low-density array system. Of the 381 miRNAs represented on the well plates, 331 miRNAs were not detected after the 35 PCR cycles. Among the remaining 50 miRNAs, upregulated and down-regulated miRNAs in MM plasma samples (expression level in the sample was 4-fold greater or lower than that of healthy volunteers) were selected. We then evaluated the rank order of expression of each miRNA by ΔCt value among all detected miRNAs.

Most of the miRNAs were downregulated in plasma samples obtained from MM patients (Figure 1a). The most striking difference of expression was found in miR-223. In addition, members of the miR-17-92a cluster (miR-19b, miR-17, miR-92a, miR-20a and miR-19a) were significantly decreased in MM
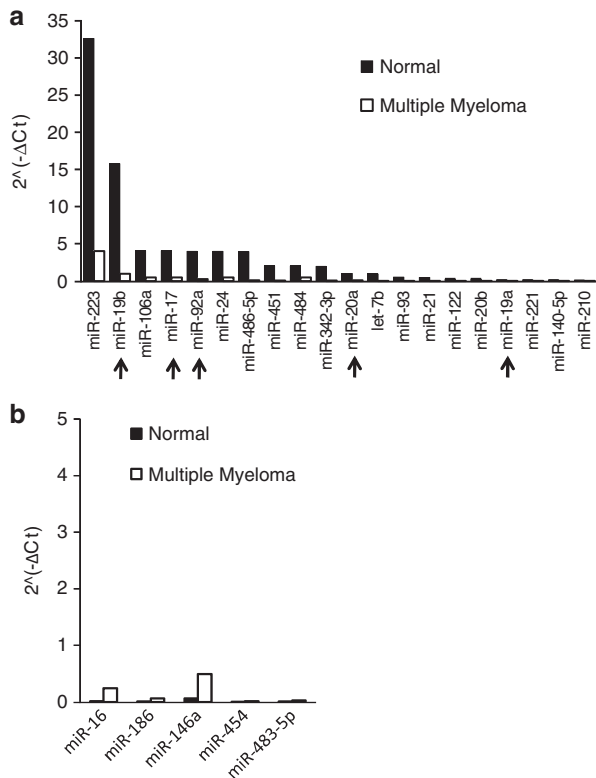
Figure 1. Identification of expressed plasma miRNAs in MM. (**a**) Downregulated miRNAs in MM (open bars). The expression level in the sample was 4-fold lower than that of normal controls (solid bars). Arrows indicate miR-17-92a polycistronic cluster. (**b**) Upregulated miRNAs in MM (open bars). The expression level in the sample was 4-fold higher than that of normal controls (solid bars).

Figure 2. Plasma miR-92a values in MM and receiver operating characteristic curve. (**a**) Plasma miR-92a level (miR-92a/miR-638) in patients with MM at the time of diagnosis. A significant downregulated plasma miR-92a level is notable in MM at diagnosis (MM-Dx) ($P < 0.0001$). No particular difference in plasma miR-92a level is evident among immunoglobulin subtypes, Bence–Jones protein type or non-secretory MM by one-way ANOVA. Bars indicate minimum to maximum plasma miR-92a levels and boxes indicate 95% confidence interval (CI). (**b**) The cut-off level of plasma miR-92a/miR-638 in all MM at diagnosis is 0.2593. The sensitivity is 91.94% (95% CI: 82.17–97.33%) and specificity is 99.12% (95% CI: 95.17–99.98%).

samples. Although we found several upregulated miRNAs in MM samples, the Ct values were generally low in both MM samples and normal controls (Figure 1b). We therefore focused on downregulation of the miR-17-92a cluster, rather than the single miRNA (that is, miR-223), because the expression of miR-17-92a cluster is essential in the lymphoid ontogeny and measured the expression level of miR-92a in a large series of patients.

Plasma miR-92a level of symptomatic MM

Plasma miR-92a levels were significantly lower in newly diagnosed MM patients ($n = 62$) compared with healthy controls (Student's *t*-test: $P < 0.0001$); the level of plasma miR-92a in symptomatic MM patients was $< 10\%$ compared with normal subjects (mean ± standard error $0.1008 ± 0.01899$ vs $1.388 ± 0.06993$; Table 1). Among the MM patients, there were no significant differences in the plasma miR-92a levels, irrespective of MM subtype ($P = 0.4472$) (Figure 2a), light chain type ($P = 0.4413$) (Supplementary Figure 1A) or International Staging System staging ($P = 0.1955$) (Supplementary Figure 1B). The presence of anemia ($P = 0.0990$), bone lesion ($P = 0.6701$), renal damage ($P = 0.4258$) or hypercalcemia ($P = 0.1989$) did not affect plasma miR-92a level at MM diagnosis (Supplementary Figure 2AD, Supplementary Table 1). Plasma miR-92a level was not correlated with beta2 microglobulin elevation ($P = 0.3675$), albumin level ($P = 0.0693$), elevated lactate dehydrogenase ($P = 0.4863$) or performance status ($P = 0.9850$) (Supplementary Table 1).

The receiver operating characteristic curve was generated by comparing the patients' ΔCt values with those of healthy volunteers. The analysis showed that miR-92a was a good marker of newly diagnosed MM (AUC = 0.9810), indicating 91.94%
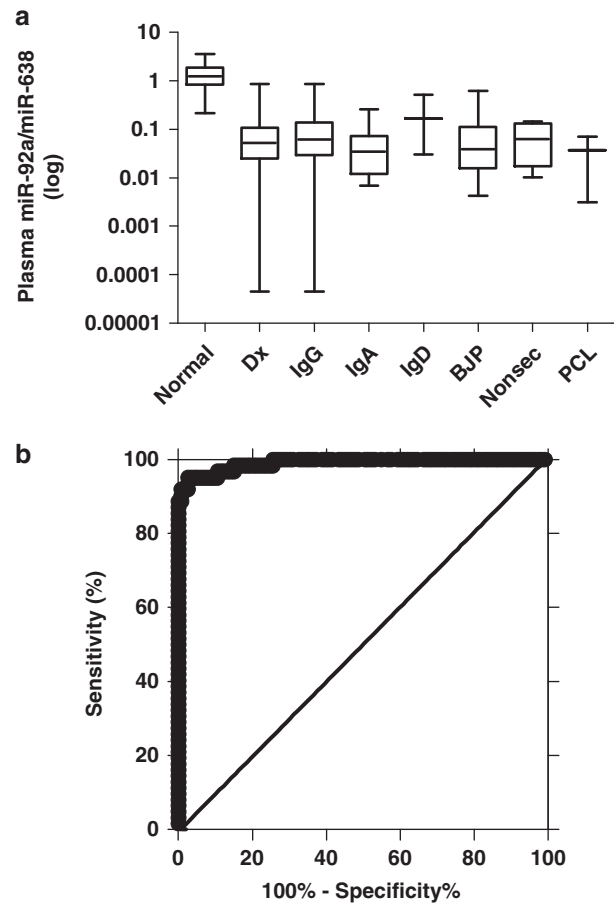
sensitivity (95% confidence interval: 82.17–97.33%) and 99.12% specificity (95% confidence interval: 95.17–99.98%) when the cut-off levels of plasma miR-92a in MM patients at diagnosis could be achieved (that is, 0.2593) (Figure 2b).

Plasma miR-92a level in the various states of MM

We next compared the plasma miR-92a level in MM patients in various clinical states (Table 1). Patients in CR ($n = 8$) had a significant increase in plasma miR-92a compared with those at diagnosis ($P < 0.0001$), and seven out of the eight patients were normalized ($\geqslant 0.2593$ cut-off level). Of the PR patients ($n = 15$), a significant increase in plasma miR-92a was notable ($P < 0.0001$), but these patients still had low plasma miR-92a compared with normal subjects ($P = 0.0033$). Although patients with stable disease ($n = 14$) had a partially normalized plasma miR-92a level, three patients had an extremely low level, similar to those of patients with newly diagnosed MM. In progressive disease patients ($n = 28$), the plasma miR-92a level again downregulated and no significant difference was notable compared with newly diagnosed MM (Figure 3a).

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Plasma miR-92a levels of MGUS and SMM patients

We also measured plasma miR-92a levels in 8 patients with SMM and 22 with MGUS (Table 1). Compared with normal subjects, patients with SMM showed no significant differences in plasma miR-92a levels ($P = 0.4642$), but they had elevated levels compared with MM patients ($P = 0.0496$) (Figure 3b). The plasma miR-92a level was significantly decreased in MGUS patients compared with normal subjects ($P < 0.0001$), but they had significantly higher plasma miR-92a levels compared with MM patients ($P = 0.0005$). No difference in plasma miR-92a levels was noted between SMM and MGUS patients ($P = 0.2959$). The plasma miR-92a levels did not correlate with duration between the time of diagnosis of MGUS and the measurement of levels in this study (data not shown).

miR-92a level in separated lymphocytes

We then compared miR-92a levels in T lymphocytes obtained from healthy subjects ($n = 21$) with those of MM patients at diagnosis ($n = 6$) or at CR/VGPR status ($n = 5$). The miR-92a level in $CD4^+$ or $CD8^+$ T lymphocytes in MM patients at diagnosis was significantly lower compared with that of healthy subjects ($CD4^+$: $P = 0.0178$; $CD8^+$: $P = 0.0092$) (Figure 4a). Comparison of miR-92a of $CD4^+$ and $CD8^+$ T lymphocytes showed a roughly linear correlation, and most MM patients in CR/VGPR status had an increased level (Figure 4b). Although no apparent correlation was evident in normal subjects, MM patients with low levels of plasma miR-92a had low levels of miR-92a expression in $CD4^+$ (Figure 4c) and $CD8^+$ T lymphocytes (Figure 4d), and three out of the five MM patients with CR/VGPR had normal levels of plasma and cellular miR-92a.

DISCUSSION

The use of plasma miRNAs as potential biomarkers is a growing research area,[12-14] and this is the first study to look at plasma miRNAs in MM. Our findings indicate the possible clinical utility of plasma miRNA level in MM patients. The recent introduction of new agents has improved the response rate and survival in patients with MM.[21] In MM treatment, the decision to start chemotherapy usually depends on the presence of clinical symptoms with high evidence level, so-called CRAB (hypercalce-mia, renal failure, anemia, bone lesion) symptoms.[22] Moreover, the therapeutic target point in MM patients has been proposed,[23] and practical guidelines for the therapeutic strategy have been helpful in clinical decision-making for MM treatment.[24,25] However, there are some MM patients for whom the timing and combination of chemotherapy are difficult to decide,[26] although high-risk MM patients can be classified.[27,28] Because, decisions regarding chemotherapy are occasionally difficult in non-secretory or asymptomatic MM patients, a novel diagnostic marker is urgently required, in addition to the conventional diagnostic tool.[21] In the current study, we demonstrated the downregulation of plasma miR-92a in symptomatic MM patients, irrespective of the presence or absence of renal damage or M-protein, suggesting that the measurement of the plasma miR-92a could be helpful for deciding when to initiate chemotherapy.

Chromosomal abnormalities and molecular alterations in MM cells have been extensively investigated, and some of them are currently incorporated into the risk analysis.[23,27,29] In addition, pathogenesis of extracellular circumstances, including angiogen-esis,[30] in MM patients is an important issue not only for understanding the biology of myeloma but therapeutic strategies as well.[21] In myeloma cells, overexpression of the miR-17-92a has been noted in the transformation from MGUS to MM.[15] Therefore, miR-17-92a expression is thought to correlate with tumor burden in MM patients. In contrast, we found no difference in plasma miR-92a levels between MGUS and SMM patients. Because the plasma miR-92a level was significantly different between patients with SMM
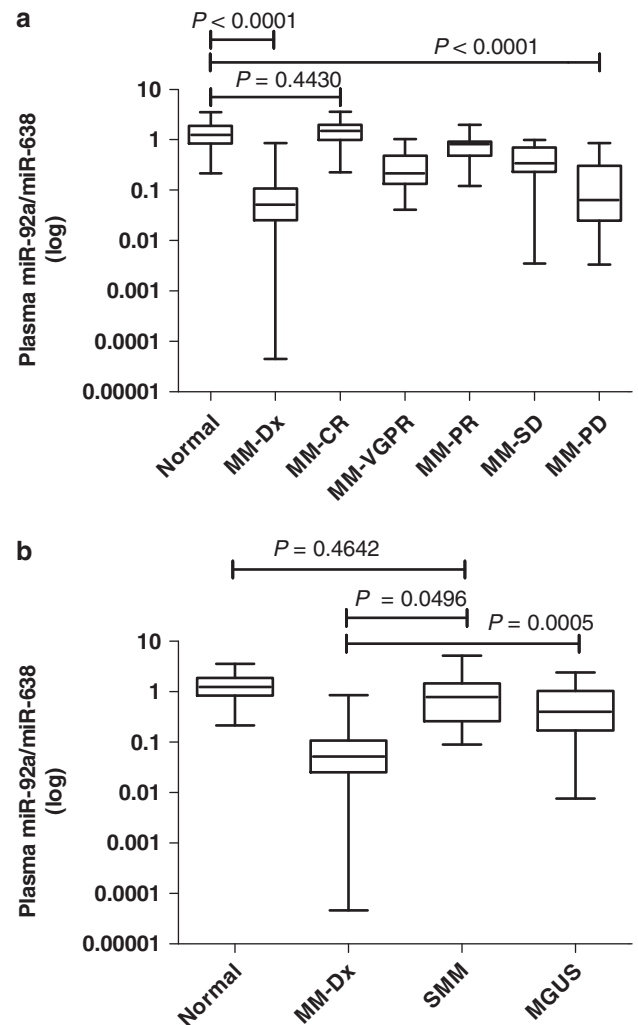
Figure 3. Plasma miR-92a expression in MM at various clinical phases and SMM or MGUS. (**a**) The downregulated plasma miR-92a level at myeloma diagnosis (MM-Dx) was normalized in CR (MM-CR) and was partially normalized at VGPR (MM-VGPR) or PR (MM-PR) phase. (**b**) Patients with SMM had a higher plasma miR-92a level than that of symptomatic myeloma ($P = 0.0496$), but they had low levels compared with normal subjects. Although some patients with MGUS had low levels of plasma miR-92a, most MGUS patients had plasma miR-92a levels similar to those with SMM. Bars indicate minimum to maximum plasma miR-92a levels and boxes indicate 95% CI.

and symptomatic myeloma, the level of plasma miR-92a level could reflect the pathological condition of patients rather than the tumor burden of myeloma cells in the body. Moreover, normalization of the plasma miR-92a level after obtaining CR suggests that the plasma miR-92a level might serve as an indicator for therapeutic response. The number of treated patients studied was small, however, and further research is needed to clarify this possibility.

Studies have shown that some circulating miRNAs in cancer-bearing patients are tumor-derived,[7] and thus such miRNAs might be used as biomarkers of cancer.[12-14] The downregulation of plasma miR-92a has been recognized in patients with hematologic neoplasia, including acute leukemia,[16,17] non-Hodgkin's lympho-ma[18] and hepatocellular carcinoma,[31] as well as in patients with cardiovascular diseases.[32] Because miR-17-92a is essential in the development and ontogeny of the lymphoid system,[33,34] we examined the correlation between miR-92a levels in separated lymphocytes and plasma. The plasma miR-92a levels was
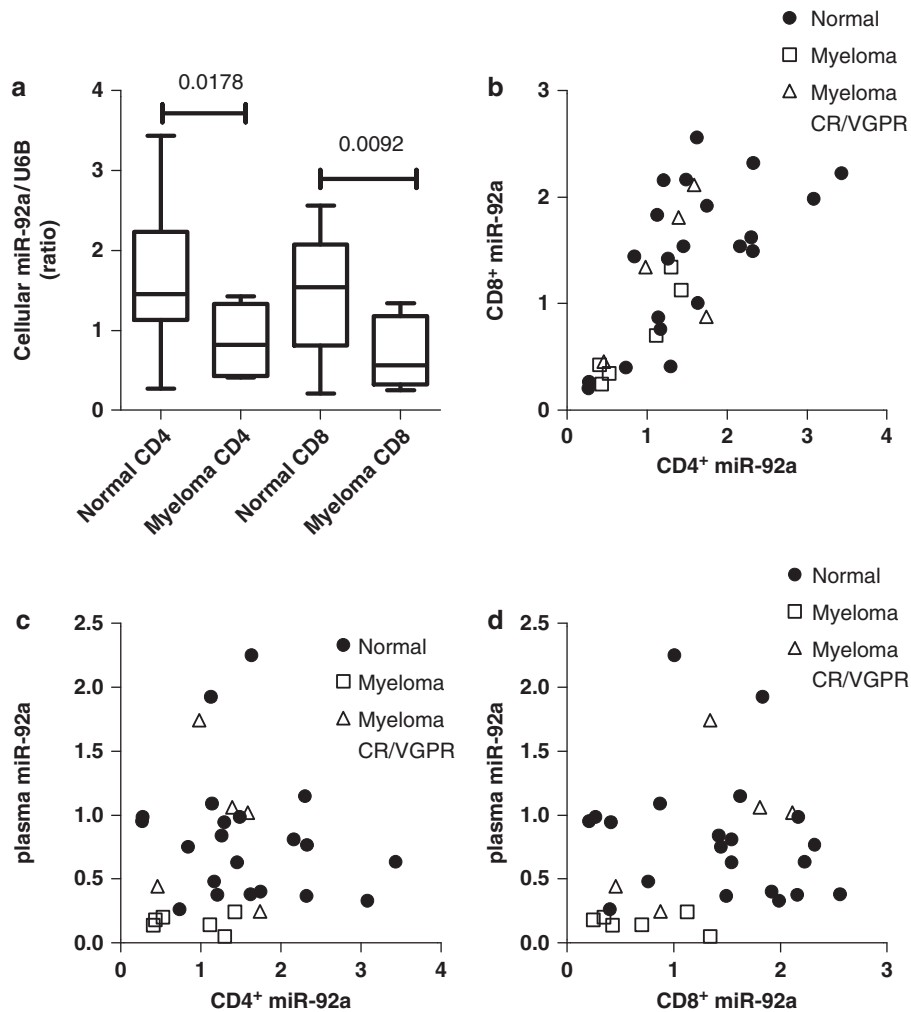
Figure 4. Cellular miR-92a in CD8+ or CD4+ lymphocytes in MM and normal subjects. (a) The CD4+ miR-92a ($P = 0.0178$) and CD8+ miR-92a ($P = 0.0092$) are significantly downregulated. (b) Comparison between miR-92a levels in CD4$^+$ and CD8$^+$ T lymphocytes in normal subjects (closed circles), MM at diagnosis (open squares) and myeloma in CR/VGPR (open triangles), showing a positive correlation. Comparison between plasma miR-92a and the cellular miR-92a of CD4$^+$ T lymphocytes (c) or CD8$^+$ T lymphocytes (d) in identical subjects. Myeloma patients with low levels of miR-92a in CD4$^+$ T lymphocytes cluster at low levels of plasma miR-92a, and some MM patients with CR/VGPR show normalization of miR-92a levels in plasma, CD4$^+$ and CD8$^+$ T lymphocytes.

correlated with the miR-92a level in T lymphocytes, especially CD8$^+$ lymphocytes. Some MM patients in CR/VGPR showed normalization of the plasma miR-92a level, in combination with upregulation of T-cell miR-92a. A recent study reported that the miR-17-92a cluster was critical for optimal T-lymphocyte co-stimulation via CD28 and was thereby essential for regulatory T function under certain conditions.[35] Moreover, it is reported that the type-1-skewing tumor microenvironment induces downregulation of miR-17-92a expression, with decreased levels of miR-17-92a noted in both CD4$^+$ and CD8$^+$ cells in tumor-bearing mice and in CD4$^+$ cells in patients with glioblastoma multiforme.[36] Together, these findings suggest that the low level of miR-92a in T lymphocytes may represent dysregulation of immunity and the downregulated plasma miR-92a level in MM patients may reflect the dysfunction of T lymphocytes in vivo. In addition to miR-17-92a downregulation, the TaqMan low-density array screening revealed downregulation of miR-223, a regulator of neutrophil proliferation and activation.[37] The ultimate downregulation of plasma miR-223 in MM patients might also be related with granulocyte dysfunction.

In conclusion, the plasma miR-92a level may serve as a biomarker for monitoring therapeutic response in MM patients and disease progression in asymptomatic MM patients. The

downregulated plasma miR-92a level may be linked to the expression level of T-cell-derived miR-92a. Although we examined a small number of patients, especially MM patients who had been treated, these initial findings suggest that plasma miR-92a levels in monoclonal gammopathy patients may be a promising parameter not only for determining disease status but also whether further treatment is required.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS
SY, KO and JHO designed the study, analyzed the data, interpreted the data and wrote the manuscript; TU and MO performed quantitative PCR analysis of plasma miRNA; KO performed statistical analysis; SY KO, KS, AI, and SI provided patient samples.

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)