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Dysregulation of miRNAs in AL amyloidosis

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Abstract

Bone marrow plasma cells (BMPCs) were purified using anti-CD138 immunomagnetic beads, from aspirates obtained with permission of the Boston University Medical Campus Institutional Review Board, from patients with immunoglobulin light chain (AL) amyloidosis and from controls. Expression levels of MicroRNAs (miRNAs) were compared by microarray; 10 were found to be increased more than 1.5-fold. These results were confirmed using stem-loop RT-qPCR for the most highly upregulated miRNAs, miR-148a, miR-26a, and miR-16. miR-16, a micro-RNA linked to other hematopoietic diseases, was significantly increased in the AL group at diagnosis, and also in treated patients with persistent monoclonal plasma cells in the bone marrow, but not in patients who achieved a hematologic remission after therapy. miR-16 can be derived from the miR-16-1/miR-15, a cluster on chromosome 13 or the miR-16-2/miR-15b cluster on chromosome 3. The expression of miR-15b was much higher than miR-15a in both AL and control BMPC, suggesting that miR-16 in plasma cells is mainly derived from miR-16-2/miR-15b. The anti-apoptosis gene BCL-2, a putative target mRNA that can be downregulated by miR-16, was expressed in BMPCs from AL patients, despite elevated levels of miR-16. Our data suggests that miRNAs are dysregulated in clonal plasma cells in AL amyloidosis and may be potentially useful as biomarkers of disease.

Keywords

Amyloidosis; plasma cell; micro-RNA; BCL-2; biomarker

Introduction

The amyloidoses are a group of diseases that involve extracellular deposition of insoluble fibrils in tissues and organs. Immunoglobulin light chain (AL) amyloidosis is the most common form of systemic amyloidosis in the US and Europe, with an incidence estimated at 5–12 cases/million/year. AL is caused by a clonal expansion of bone marrow plasma cells (BMPCs) and concomitant over-production of monoclonal ALs that misfold and aggregate [1]. While AL shares some common genetic events with the plasma-cell malignancy multiple myeloma (MM), there are clear differences between MM and AL plasma cells. Compared to MM, the percentage of BMPCs are lower in AL; lytic bone lesions do not occur [2]; cell lines cannot be generated without ectopically introducing an oncogene; and lastly, the λ 6 light chain gene is frequently used in AL and rarely utilized in MM plasma cells [3]. Only about 10–15% of MM patients develop AL, and AL rarely progresses to MM [4]. The pathogenesis of BMPC clonal expansion and the molecular mechanism of amyloid fibril formation in AL are not fully understood.

The diagnosis of AL is established by evidence of systemic deposition of amyloid fibrils along with proof of the clonal expansion of BMPC and production of monotypic immunoglobulins (Igs) [5]. Due to the pleomorphic clinical presentation of AL, patients are frequently not diagnosed until significant organ impairment has occurred; the median survival of untreated AL patients is approximately 10–14 months from diagnosis [6,7]. Currently, the goal of treatment for AL is eradication of the clonal BMPC. This can be achieved using high-dose melphalan and autologous bone marrow stem cell transplantation, which leads to durable remissions and prolonged survival [8]. Combinations of oral melphalan and dexamethasone [9], and novel agents including immunomodulatory agents [8,10] and proteasome inhibitors [11,12] have also proved effective. However, these treatments all have associated toxicities. Understanding the molecular pathogenesis of AL amyloidosis and developing targeted treatments are important goals. Identification of key regulatory genes in clonal AL BMPC may provide tools for early diagnosis and targets for treatment.

MicroRNAs have recently been identified as non-coding RNAs that play regulatory roles in development and in tumorigenesis [13,14]. Mature miRNAs, approximately 22 nucleotides in length, are generated from long primary transcripts (pri-MicroRNA), and usually derived from introns or non-coding regions of the genome. The miRNAs guide the RNA-induced silencing complex (RISC) to the cognate messenger RNA (mRNA) target leading to translational repression or degradation of mRNA [15]. miRNAs can function as oncogenes or tumor suppressors depending upon their targets [16]. Interestingly, more than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites, underscoring their potential importance in malignancy [17]. Indeed, several miRNAs have been shown to be directly involved in human cancers, including lung, breast, brain, liver, colon, and hematological malignancies [13,18]. For instance, miR-15a and miR-16-1 have been found in the minimally deleted region of 13q14 in malignant cells from patients with chronic lymphocytic leukemia (CLL), and their expression is reduced in those cells [19]. It has been demonstrated that miR-16 can downregulate BCL-2 expression [20–22]. In normal B-cells, there are miRNAs that are critical for differentiation, such as miR-155 which

regulates Ig class switching and is required for the terminal differentiation of antibody-producing B-cells. B-cells lacking miR-155 have attenuated extrafollicular and germinal center responses and fail to produce high-affinity IgG1 antibodies [23–25]. Thus, miRNAs regulate B-cell differentiation, function, and transformation. Collectively, this information provides the rationale for our hypothesis that miRNAs are dysregulated in BMPC derived from patients with AL amyloidosis. Abnormal levels of miRNAs may play an important role in the pathogenesis of AL and may be useful in the diagnosis and tracking of minimal residual disease and/or as potential therapeutic targets.

Methods

Subjects

We collected bone marrow aspirates, with approval of the Boston University Medical Campus Institutional Review Board, from patients referred for evaluation of amyloidosis from May 2008 to April 2009. Thirty-three patients were newly diagnosed with AL amyloidosis; all had proof of systemic amyloidosis, with a plasma-cell dyscrasia in the bone marrow, and a monoclonal gammopathy demonstrated by an abnormal κ/λ free light chain ratio and/or a monoclonal immunoglobulin on serum or urine immunofixation electrophoresis (SIFE or UIFE). Twenty-five patients had undergone prior therapy. Non-AL controls included five patients with no amyloid or plasma cell disease, 10 patients with hereditary or amyloid A (AA) amyloidosis, and 16 patients with localized amyloidosis. AL and control groups were similar in age (range, 35–84 years) and sex distribution (male to female ratio, 1.5:1).

BMPC isolation

Mononuclear cells in fresh bone marrow aspirates were enriched and red blood cells were removed by differential centrifugation on Ficoll-Hypaque medium (Amersham, specific gravity of 1.066), according to the manufacturer's instructions. After two washes with phosphate buffered saline (PBS), the mononuclear cells were resuspended in 75 μ L of buffer A (PBS/0.5 mM EDTA) and incubated with 25 μ L of immunobeads conjugated with human CD138-specific antibody (Miltenyi Biotec, CA) at 4–8°C for 20 min. Excess immunobeads were removed by washing the cells twice with buffer A. The washed cells were resuspended in 500 μ L of buffer A and loaded onto an MS column (Miltenyi Biotec) in a magnetic field. The column was washed three times with buffer A to remove CD138[−] cells. The column was removed from the magnetic field and the retained cells were eluted with 1 mL of buffer B (PBS containing 0.5% bovine serum albumin).

Flow cytometry

Five percent of the starting mononuclear cells and the purified plasma cells were resuspended in 90 μ L of buffer B and incubated with 10 μ L of PE-conjugated CD138-specific antibody (Miltenyi Biotec) at 4°C for 30 min. The cell pellets were washed twice with buffer B. The labeled cells were resuspended in 500 μ L of buffer B and analyzed in the flow cytometer.

MicroRNA array

MiRNA expression was determined using the miRXplore Microarray platform (Miltenyi Biotec). A pooled sample containing 2 µg of total RNA from four AL patients (500 ng each) was labeled with Hy5, a red fluorescent dye. Similarly, a control pooled sample of total RNA from four non-AL patients (one had localized amyloidosis, one AL amyloidosis, one AA amyloidosis, and one no amyloid disease) was prepared and labeled with Hy3, a green fluorescent dye. The labeled samples were hybridized to the microarray containing capture probes for more than 800 human miRNAs and 150 human viral miRNAs spotted in four subgrids, listed in the miRXplore Microarray miRBase. The relative signal intensities were represented as the $\log_2$ -mean of four Hy5/Hy3 ratios for each miRNA. A ratio of more than 1.5 or less than 0.67 was considered as significantly increased or decreased expression for the corresponding miRNA.

Quantitative TaqMan MicroRNA assay

RNA was isolated by using the *mirVana*TM miRNA Isolation Kit (Applied Biosystems, CA; part number AM1560). MicroRNA quantification was performed by stem-loop reverse quantitative real-time polymerase chain reaction (RT-qPCR) [26] using TaqMan[®] MicroRNA Reverse Transcription Kit (Applied Biosystems) for RT; TaqMan[®] Universal PCR Master Mix (Applied Biosystems) for qPCR; and MicroRNA qPCR kit containing RT primer, PCR primers, and probe for each individual miRNA (mirVana-miRNA detection kit; hsa-miR-16 assay, hsa-miR-15a assay, hsa-miR-15b assay, hsa-miR-148a assay, hsa-miR-26a assay, and RNU6B assay; Applied Biosystems). The experimental conditions followed the recommendations provided by the manufacturer. Reactions were performed in triplicate and expression of MicroRNA was presented as 2^{-CT} , where $CT = CT_{\text{target miRNA}} - CT_{\text{reference gene}}$. U6B RNA was used as a reference. The differences in miRNA levels between AL and control samples were analyzed using a Student's *t*-test to assess statistical significance.

Immunostaining

Bone marrow biopsies were fixed in B5, decalcified and embedded in paraffin, and cut into 5 µm sections. Immunohistochemistry was performed using Ventana Medical Systems' BenchMark[®] XT slide stainer (Tucson, AZ). Briefly, sections were subjected to antigen retrieval after deparaffinization and incubated with pre-diluted monoclonal mouse anti-Bcl2 (Ventana, Tucson, AZ) for 32 min at 37°C. After incubating with secondary antibody (Amplification Kit; Ventana), the bound complexes were visualized with the ultraViewTM Universal DAB Detection Kit (Ventana), and the slides were counterstained with hematoxylin and blueing reagent for 12 min each. Processed sections from a tonsil biopsy specimen were used as the positive control and the specificity of the primary antibody was confirmed using a pre-diluted negative control reagent (Ventana).

Results

Purification of BMPCs from patients with AL amyloidosis and controls

The clonal BMPCs in AL patients constitute a minor population (generally 5–20%) of the nucleated cells in bone marrow. In patients without a plasma-cell disorder, polyclonal plasma cells generally represent less than 5% of the nucleated cells. Even in disease, plasma cells are terminally differentiated, non-dividing cells that cannot be easily cultured *ex vivo*. It is critical to isolate fresh BMPCs with high purity in order to study the unique biology of plasma cells with minimal or no interference from other types of bone marrow cells. We established a protocol to isolate these cells from clinical samples using magnetic antibody-microbeads specific for CD138, a marker for BMPC. Using this technique, BMPC of more than 90% purity were typically obtained even from normal subjects with less than 5% plasma cells in the starting aspirate. A typical example of a flow cytometry analysis of plasma cells from a bone marrow aspirate of a non-AL patient whose percentage was 0.74% initially (Figure 1A) and 92.5% following enrichment by magnetic immuno-microbead purification (Figure 1B) is shown. The fall-through from the MS column contained less than 0.1% CD138⁺ cells (Figure 1C). Even higher percentages of plasma cells were obtained from aspirates from patients with AL. The purity of the plasma cells was confirmed by immunostaining (Figure 1D, 1E).

Identification of dysregulated miRNAs in BMPCs of AL patients

Having isolated BMPCs at more than 90% purity, we next sought to identify miRNAs that are differently expressed in the BMPCs of AL patients. Pooled samples from four AL patients and four non-AL subjects as controls were used in the screen, to reduce the number of false positives from the variability of individual samples. Ten miRNAs that were upregulated at least 1.5-fold in BMPCs of AL patients are listed in Table I. No significantly downregulated miRNAs were found in this screen. We used RT-qPCR to further examine levels of three miRNAs which appeared to have the highest upregulation in AL by microarray analysis, miR-148a, miR-26a, and miR-16. Confirmation of the microarray data was carried out using individual BMPC samples from a panel of 12 AL patients and eight controls. The fold increase in expression of miR-148a, miR-26a, and miR-16 was 7.0, 4.4, and 3.6 by RT-qPCR compared to 8.9, 4.7, and 7.6 by microarray (Table II). Interestingly, there was a high correlation, $R^2 = 0.97$ (Figure 2), between miR-26a and miR-148a expression, while the R^2 for miR-148a with miR-16 and miR-26a with miR-16 were 0.43 and 0.53, respectively.

Upregulation of MicroRNA-16 in BMPCs from AL patients

It has been shown that miR-16 is decreased in CLL cells with chromosome 13 deletions. Furthermore, chromosome 13 deletions occur in BMPCs of MM patients. To confirm the data above indicating that miR-16 is elevated in BMPC from patients with AL, we extended our analysis to a total of 33 AL patients and 31 controls and found that there was a significant difference between the groups ($p < 0.0001$). The mean ($\pm$ SD) values for the AL and control samples were 43.8 ($\pm$ 29.4) and 15.5 ($\pm$ 17), respectively (Figure 3A). In the control samples, the mean levels of miR-16 in BMPCs from patients with AA and hereditary (ATTR) amyloidosis, localized amyloidosis, and non-amyloidosis controls were 11.7, 18.6,

and 9.0, respectively. We next investigated whether treatments that effectively eradicate the clonal plasma cells in AL patients would also reduce the miR-16 expression level. As shown in Figure 3B, the mean level of miR-16 (16.5 ± 11.1) in the seven AL patients who achieved a complete hematologic response after anti-plasma cell treatment was similar to that of control group (15.5 ± 17.5) shown in Figure 1A. However, the mean level remained high (59.9 ± 45.6) in the 18 patients with persistent disease after treatment and was comparable to the mean miR-16 level in newly diagnosed AL patients (43.8 ± 29.4). There was a significant difference in miR-16 levels between the patients with persistent disease and those with a complete response (p -value = 0.0029). Examining these data more closely, it can be seen that the miR-16 levels in the patients with persistent disease after treatment were distributed into two distinct groups; the miR-16 level in the lower group was similar to that of the complete response group and the controls. As these patients are followed over time, it will be interesting to see if the miR-16 levels correlate with risk of progression of disease.

The level of miR-15b is higher than miR-15a in BMPCs

Mature miR-16 consists of two identical miRNAs, miR-16-1 and miR-16-2. MicroRNA-16-2 is clustered with miR-15b on chromosome 3 while miR-16-1 is clustered with miR-15a on chromosome 13. MicroRNA-15b differs from miR-15a in four nucleotides and can be distinguished by RT-qPCR. We analyzed miR-15a and miR-15b levels in BMPCs from 12 AL patients and nine controls. As shown in Figure 4, the expression levels of miR-15b are 2–10-fold higher than miR-15a in all cases. This variation in amounts of miR-15a and miR-15b was not an artifact of a difference in amplification efficiency, as synthetic miR-15a and 15b were amplified equally (data not shown). These results raise the possibility that miR-16 in BMPCs is mostly derived from chromosome 3 and not chromosome 13.

BCL-2 protein is expressed in AL BMPCs with high miR-16 expression

It has been postulated that miR-16 induces cell death by reducing BCL-2 expression, and loss of miR-16 in some lymphoid malignancies results in upregulation of BCL-2 and inhibition of apoptosis. We used immunostaining to qualitatively compare BCL-2 levels in bone marrow core biopsies from two controls, three AL patients with low miR-16 levels, and three AL patients with high miR-16 levels. As shown in Figure 5, there was no difference in the intensity of BCL-2 staining in core biopsies from the three patients with high miR-16 levels (Figure 5H, 5I), compared to the three patients with low miR-16 levels (Figure 5D, 5E, 5F) or controls (Figure 5B, 5C). Therefore, there does not appear to be an inverse correlation between miR-16 levels and BCL-2 protein levels in BMPCs of AL patients.

Discussion

There is increasing evidence that MicroRNAs play important roles in tumorigenesis [14,16,18]. Dysregulation of MicroRNAs has been reported in many solid tumors and hematological malignancies [27–29] including MM [30–32], but to our knowledge, the role of MicroRNA dysregulation in AL amyloidosis has not been investigated. MicroRNAs expressed in highly purified BMPCs from patients with systemic AL amyloidosis were compared to a variety of controls, including patients with no form of amyloidosis or plasma-

cell disease, patients with hereditary or AA amyloidosis, and patients with localized amyloidosis. There were no significant differences between expressions in these various control populations. Of 10 upregulated miRNAs, the three most highly overexpressed in the AL BMPCs were confirmed using stem-loop RT-qPCR. Two of the three, miR-148a and miR-26a, were highly correlated between samples, suggesting that these MicroRNAs are co-regulated. A third, miR-16, was quite variable in individual samples, but averaged three-fold higher levels compared to controls. We focused our attention on miR-16 because of its reported association with other hematologic disorders.

In CLL cells, miR-16 has been shown to be a negative regulator of BCL-2 with loss of activity through deletion of chromosome 13q14, the region harboring a putative tumor suppressor [19,33,34]. A similar deletion has been reported in prostate cancer [20,35,36]. Deletion of 13q14 is also found in BMPCs from 30–50% of patients with MM [37–39] and is a poor prognostic factor [40,41], while the same chromosome abnormality in CLL is associated with a better prognosis [42]. AL BMPCs can also have deletion of 13q [43,44]. However, low levels of miR-16 have not been found in MM [30,32,45]. In fact, the supplemental data from Pichiorri et al. demonstrated increased miR-16 and miR-15 levels in samples from patients with monoclonal gammopathies of undetermined significance and MM [30] and this has been confirmed in a recent report, that also failed to find a correlation between miR-16 or miR-15a expression and chromosome 13q deletion status [46]. These observations, and our data, suggest that the regulation of miR-16 levels in BMPCs could be different from that in CLL cells. The mechanisms involved in the regulation of miRNA expression levels are poorly understood. The level of a given MicroRNA can be regulated by transcription of pri-MicroRNA, by processing the pri-MicroRNA into a pre-MicroRNA and then into the mature form, by exporting the mature miRNA from the nucleus into the cytoplasm, and finally by degradation [19]. Mature miR-16 is made up of two identical miRNAs, miR-16-2 that is in a cluster with miR-15b located on chromosome 3 [47], and miR-16-1 that is clustered with miR-15a on chromosome 13 [19]. We found that the level of miR-15b is much higher than miR-15a in BMPCs (Figure 4). This could be due to more an active transcription at the miR-16-2/miR-15b cluster on chromosome 3, more rapid maturation or transportation of miR-15b, or higher stability of miR-15b. In the former case, deletion of chromosome 13 would not affect the levels of miR-16 in BMPCs. This, taken together with the correlation between the levels of miR-16 and the deletion of chromosome 13 in CLL cells [47], raises the interesting possibility that there exists a cell type-specific regulation of the gene cluster used to generate miR-16.

BCL-2 is an anti-apoptotic gene that plays an important role in the molecular pathogenesis of many hematologic and solid cancers [48,49]. MicroRNA-16 can negatively regulate BCL-2 in CLL cells, and it has been postulated that the loss of miR-16 causes upregulation of BCL-2 [22], although not all studies support this [33,34]. We found strong expression of BCL-2 protein in AL BMPCs by immunohistochemistry, in spite of high levels of miR-16. Thus, in AL BMPCs, miR-16 expression is not sufficient to suppress BCL-2. miR-16 has 8778 potential targets in the genome (<http://www.microrna.org/>) and identification of key targets regulated in AL amyloidosis plasma cells will require considerable further study.

Conclusions

We found that miR-16 and several other miRs are dysregulated and expressed at increased levels in BMPCs from AL patients and that miR-16 levels were reduced in patients who had responded to anti-plasma cell chemotherapy, but not in patients who had persistent disease. This supports a potential functional role of miR-16 elevation in AL amyloidosis, and its potential as a biomarker in some patients with this disease.

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Figure 1.

Purity of BMPCs isolated using immunomagnetic beads directed against CD138.

Fluorescence-activated cell sorting (FACS) analysis of CD138⁺ plasma cells in unpurified bone marrow (A) identifies 0.74% of total nucleated cells in region 3 (R3) as plasma cells. After immunomagnetic bead separation, the BMPCs are enriched to 92.5% purity (B), with <0.2% BMPCs present in the non-binding cell population (C). FACS data were confirmed comparing CD138 immunostaining of the bone marrow biopsy specimen (D) and the BMPC population eluted from the immunomagnetic beads (E).

Figure 2.

The correlation between miR-148a and miR-26a from a panel of samples containing 12 AL patients and nine controls; $R^2 = 0.94$. Controls for this experiment included four patients with localized amyloidosis, two with no amyloidosis, and three patients with AF or AA amyloidosis.

Figure 3.

MicroRNA-16 derived from BMPCs is significantly increased in untreated AL patients and decreased in AL patients who have had a complete hematologic response to treatment. (A) By stem-loop RT-PCR, the mean levels of miR-16 levels were significantly higher in 33 AL patients (circles) compared to 31 controls (square), (43.8 ± 29.4 versus 15.51 ± 17.45 , $p < 0.001$). (B) By stem-loop RT-PCR, miR-16 levels in 18 AL patients who had persistence of clonal-plasma-cell dyscrasia post-treatment (circles) were higher than those of seven patients who had a complete response (squares) to treatment (59.93 ± 45.566 versus 16.495 ± 11.14 , $p = 0.0029$). The large filled circle and bars represent means and SDs.

Figure 4.

MicroRNA-16 is mainly derived from the miR-16-2/miR-15b cluster located on chromosome 3. The ratio of miR-15b/miR-15a from 12 AL patients (filled circles) and nine controls (open circles) are shown. There was no significant difference between the two groups ($p = 0.79$). The control group is the same as that used for Figure 2.

Figure 5.

BCL-2 protein is present in AL BMPCs in spite of the elevation of miR-16. Analysis by immunohistochemistry shows staining in the cytoplasm for BCL-2 protein (brown peroxidase reaction). Slides were counterstained with hematoxylin and eosin. (A) A lymph node follicle is shown. B lymphocytes in the follicular center are negative for BCL-2 and T-cells in the marginal zone are positive for BCL-2. Bone marrow biopsies from two controls [(B) patient with no amyloidosis; (C) patient with localized amyloidosis], three AL patients with low miR-16 (D–F), and three AL patients with high level of miR-16 (G–I) are shown. The number in the right lower corner of each marrow biopsy specimen is the relative miR-16 level.

Table I

MicroRNAs upregulated more than 1.5-fold in pooled AL BMPCs compared with controls (see text for details), as determined by microarray screening.

miRNA	Ratio
HAS-MIR-148a	8.9
HAS-MIR-16	7.6
HAS-MIR-26a	5.6
HAS-MIR-29b	4.7
HAS-MIR-29c	3.0
HAS-MIR-29a	2.8
HAS-MIR-22	2.6
HAS-LET-7g	2.5
HAS-LET-7f	1.8
HAS-MIR-1274b	1.7

Table II

The upregulation of miRNAs with the highest levels by microarray was confirmed by stem-loop RT-PCR. Means in AL BMPCs were compared with controls (Ctrl) by Student's *t*-test.

	AL (<i>n</i> = 12)	Ctrl (<i>n</i> = 8)	<i>p</i> -Value
miR-148a	7.0	1.0	0.01
miR-26a	4.9	1.1	0.01
miR-16	36.2	10.0	0.002