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# MicroRNA expression in multiple myeloma is associated with genetic subtype, isotype and survival

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## Abstract

**Background:** MicroRNAs are small RNA species that regulate gene expression post-transcriptionally and are aberrantly expressed in many cancers including hematological malignancies. However, the role of microRNAs in the pathogenesis of multiple myeloma (MM) is only poorly understood. We therefore used microarray analysis to elucidate the complete miRNome (miRBase version 13.0) of purified tumor (CD138^{+}) cells from 33 patients with MM, 5 patients with monoclonal gammopathy of undetermined significance (MGUS) and 9 controls.

**Results:** Unsupervised cluster analysis revealed that MM and MGUS samples have a distinct microRNA expression profile from control CD138^{+} cells. The majority of microRNAs aberrantly expressed in MM (109/129) were up-regulated. A comparison of these microRNAs with those aberrantly expressed in other B-cell and T-cell malignancies revealed a surprising degree of similarity (~40%) suggesting the existence of a common lymphoma microRNA signature. We identified 39 microRNAs associated with the pre-malignant condition MGUS. Twenty-three (59%) of these were also aberrantly expressed in MM suggesting common microRNA expression events in MM progression. MM is characterized by multiple chromosomal abnormalities of varying prognostic significance. We identified specific microRNA signatures associated with the most common IgH translocations (t(4;14) and t(11;14)) and del(13q). Expression levels of these microRNAs were distinct between the genetic subtypes (by cluster analysis) and correctly predicted these abnormalities in > 85% of cases using the support vector machine algorithm. Additionally, we identified microRNAs associated with light chain only myeloma, as well as IgG and IgA-type MM. Finally, we identified 32 microRNAs associated with event-free survival (EFS) in MM, ten of which were significant by univariate (logrank) survival analysis.

**Conclusions:** In summary, this work has identified aberrantly expressed microRNAs associated with the diagnosis, pathogenesis and prognosis of MM, data which will prove an invaluable resource for understanding the role of microRNAs in this devastating disease.

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## Background

Multiple myeloma (MM) is a plasma cell (PC) malignancy with an annual incidence of over 14,000 cases in the US alone. MM is essentially an incurable disease with a median survival of ~3 years that accounts for nearly 2% of deaths from cancer and about 20% of deaths from hematological cancers [1]. Newer therapies, however, are resulting in improvements in the median survival [2]. Recent advances in molecular and genetic research have lead to the realization that MM, although defined histologically as a single entity, encompasses a wide range and frequently complex mixture of genomic abnormalities which differ in both their molecular pathogenesis and prognostic significance. The recent discovery of short non-coding RNA molecules that regulate gene expression post-transcriptionally, known as

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microRNAs, represent yet another level of complexity in our understanding of gene regulation and as such could further our understanding of the pathogenesis of MM.

MicroRNAs have been demonstrated to have diagnostic and prognostic potential in cancer [3-7] and it has been suggested that microRNA expression profiling can distinguish cancers according to both the cellular nature and the developmental stage of the tumor with a greater degree of accuracy than traditional gene expression analysis [8]. There is increasingly strong evidence that microRNAs are involved in the pathogenesis of many cancers including B and T cell lymphomas [9,10]. There is however little known about the role that microRNAs play in MM.

Therefore we undertook a comprehensive study using microarray technology to elucidate the complete miR-Nome (miRBase version 13.0) of purified tumor (CD138$^+$) cells from the bone marrow of 33 MM and 5 MGUS patients (and 9 controls). In order to investigate microRNA expression in different genetic subtypes MM cases were classified cytogenetically by FISH. These data were then correlated with genetic subtype and clinical parameters.

## Results

### Most aberrantly expressed microRNAs associated with MM are up-regulated

We elucidated the complete (miRBase v.10.1) microRNA profile of CD138$^+$ plasma B-cells from bone marrow of 33 MM patients, 5 MGUS, 9 controls, and 4 well established MM cell lines (NCI-H929, JJN3, Thiel and RPMI-8226). Unsupervised cluster analysis revealed that MM samples and MM cell lines have a distinct microRNA profile from counterpart controls (Figure 1A). Furthermore, although MGUS samples did not cluster together, they had a microRNA profile more similar to MM samples (and cell lines) than controls.

To identify microRNAs that are aberrantly expressed in MM patient samples we compared expression levels with controls by ANOVA. This resulted in the identification of 129 microRNAs ($P < 0.05$), only 20 (15%) of which were down-regulated in MM samples (Table 1). Of these 29 and 24 were previously identified as being aberrantly expressed in MM by the studies of Zhou et al [11] and Pichiorri et al [12] respectively. In order to validate the microarray data, eight microRNAs chosen for their previous association with MM or other hematological malignancies were measured by qRT-PCR. These data were consistent with the microarray results (Figure 2). Many microRNAs are encoded in clusters, and members of these clusters often exhibit the same pattern of expression [13]. A lower proportion of MM-associated microRNAs (42/109 (38%); Table 1) were encoded in clusters than generally observed (215/474
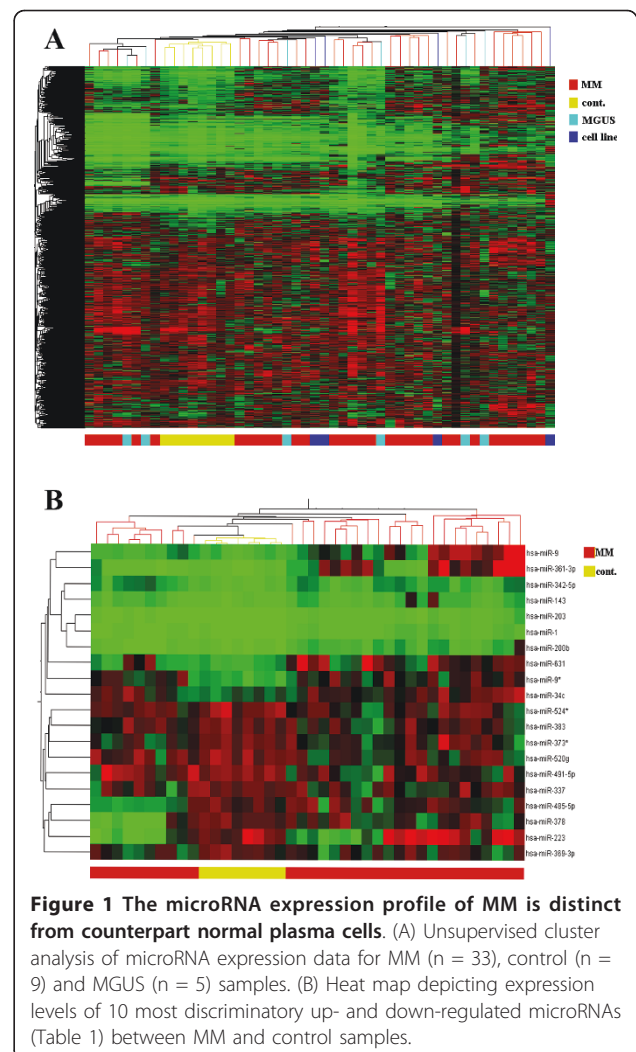


**Figure 1 The microRNA expression profile of MM is distinct from counterpart normal plasma cells**. (A) Unsupervised cluster analysis of microRNA expression data for MM (n = 33), control (n = 9) and MGUS (n = 5) samples. (B) Heat map depicting expression levels of 10 most discriminatory up- and down-regulated microRNAs (Table 1) between MM and control samples.

(45%)) (source- http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Cluster.cgi).

Expression levels of the 129 MM-associated microRNAs were distinct between MM and control samples (Additional file 1 Figure S1). Indeed using only expression levels of the ten most discriminatory (by $P$-value) up- and down-regulated microRNAs distinguished samples by cluster analysis (Figure 1B) and correctly predicted samples as either MM or control for 36/41 (88%) samples using the leave-one-out cross-validation support vector machine (SVM) algorithm.

### The microRNA profile of MGUS patient samples is distinct from MM and controls

MGUS is generally believed to represent a pre-malignant form of MM [14]. To examine microRNA expression in this condition we initially compared the profile of PC cells from MGUS patients with that of controls. Thirty-nine microRNAs were identified as being

**Table 1 MicroRNAs differentially expressed ($P < 0.05$) between MM (n = 33) and controls (n = 9) depicting chromosomal location and where relevant their expression within a microRNA cluster.**

| Order | microRNA | adj. *P* value | Fold change | Chromosome | Cluster |
|---|---|---|---|---|---|
| 1 | *miR-1* | 5.41E-06 | 3.15 | 20q13.33/18q11.2 | 1-1, 133a |
| 2 | *miR-203* | 2.09E-05 | 2.64 | 14q32.33 | - |
| 3 | *miR-342* | 2.25E-05 | 3.94 | 14q32.2 | - |
| 4 | *miR-631* | 2.25E-05 | 4.24 | 15q24.2 | - |
| 5 | *miR-200a* | 3.17E-05 | 5.50 | 12p13.31 | 141-200c |
| 6 | *miR-34c* | 5.95E-05 | 3.31 | 11q33.1 | 34b-34c |
| 7 | *miR-361* | 5.95E-05 | 12.66 | Xq21.2 | - |
| 8 | *miR-9** | 7.64E-05 | 4.48 | 1q22/5q14.3/15q26.1 | - |
| 9 | *miR-200b* | 8.52E-05 | 4.97 | 12p13.31 | 141-200c |
| 10 | *miR-9* | 8.52E-05 | 5.07 | 1q22/5q14.3/15q26.1 | - |
| 11 | *miR-151* | 1.19E-04 | 2.67 | 8q24.3 | - |
| 12 | ***miR-218*** | 1.42E-04 | 3.96 | 4p15.31/5q34 | - |
| 13 | *miR-28-3p* | 1.90E-04 | 2.52 | 3q28 | - |
| 14 | ***miR-200c*** | 2.10E-04 | 5.26 | 12p13.31 | 141-200c |
| 15 | ***miR-21*** | 2.72E-04 | 2.45 | 17q23.1 | - |
| 16 | *miR-378* | 2.72E-04 | 2.96 | 5q33.1 | - |
| 17 | *miR-548d-5p* | 2.72E-04 | 3.10 | 8q24.13/17q24.2 | - |
| 18 | *miR-621* | 2.72E-04 | 9.64 | 13q14.11 | - |
| 19 | *miR-140-5p* | 3.91E-04 | 2.78 | 16q22.1 | - |
| 20 | *miR-634* | 4.77E-04 | 2.25 | 17q24.2 | - |
| 21 | *miR-616* | 6.16E-04 | 2.69 | 12q13.3 | - |
| 22 | ***miR-130a*** | 7.81E-04 | 2.48 | 11q12.1 | - |
| 23 | *miR-593* | 7.81E-04 | 3.29 | 7q32.1 | - |
| 24 | *miR-708* | 7.81E-04 | 3.32 | 11q14.1 | - |
| 25 | *miR-200a** | 7.81E-04 | 4.08 | 12p13.31 | 141-200c |
| 26 | *miR-340* | 9.03E-04 | 2.41 | 5q35.3 | - |
| 27 | *miR-760* | 1.12E-03 | 2.44 | 1p22.1 | - |
| 28 | *miR-885-3p* | 1.39E-03 | 2.55 | 3p25.3 | - |
| 29 | *miR-590-3p* | 1.42E-03 | 3.50 | 7q11.23 | - |
| 30 | *miR-885-5p* | 1.61E-03 | 3.34 | 3p25.3 | - |
| 31 | ***miR-221*** | 2.36E-03 | 2.85 | Xp11.3 | 221-222 |
| 32 | *miR-7* | 2.36E-03 | 3.66 | 9q21.32/15q26.1/19p13.3 | - |
| 33 | ***miR-188-5p*** | 2.94E-03 | 2.18 | Xp11.23 | 532-660 |
| 34 | *miR-338* | 2.94E-03 | 2.35 | 17q25.3 | 338-657 |
| 35 | *miR-222* | 2.94E-03 | 2.47 | Xp11.3 | 221-222 |
| 36 | *miR-99a* | 2.94E-03 | 3.47 | 19q13.33 | 99b-125a |
| 37 | *miR-891a* | 3.06E-03 | 2.31 | Xq27.3 | 890-891 |
| 38 | *miR-452* | 3.77E-03 | 2.57 | Xq28 | 452-224 |
| 39 | *miR-98* | 4.09E-03 | 3.04 | Xp11.22 | 98-let-7f |
| 40 | *miR-629* | 4.09E-03 | 3.14 | 15q23 | - |
| 41 | *miR-515-3p* | 4.11E-03 | 5.90 | Xq27.3 | - |
| 42 | ***miR-192*** | 4.20E-03 | 2.44 | 11q13.1 | 192-194 |
| 43 | *miR-454* | 4.36E-03 | 2.82 | 17q22 | - |
| 44 | *miR-151-3p* | 4.59E-03 | 2.22 | 8q24.3 | - |
| 45 | *miR-141* | 5.20E-03 | 3.37 | 12p13.31 | 141-200c |
| 46 | *miR-128b* | 5.35E-03 | 2.25 | 2p21.3/3p22.3 | - |
| 47 | *miR-1227* | 5.64E-03 | 2.82 | 19p13.3 | - |
| 48 | *miR-128a* | 6.51E-03 | 2.82 | 2p21.3/3p22.3 | - |
| 49 | *miR-205* | 6.94E-03 | 3.15 | 1q32.2 | - |
| 50 | *miR-27b* | 7.30E-03 | 2.81 | 9q22.32 | 23b-24 |

**Table 1 MicroRNAs differentially expressed (P ?<? 0.05) between MM (n = 33) and controls (n = 9) depicting chromosomal location and where relevant their expression within a microRNA cluster.** *(Continued)*

| | | | | | |
|---|---|---|---|---|---|
| 51 | miR-608 | 7.67E-03 | 2.38 | 10q24.31 | - |
| 52 | miR-432 | 7.99E-03 | 2.41 | 14q32.2 | 337-136 |
| 53 | miR-220 | 8.08E-03 | 2.86 | Xq25 | - |
| 54 | **miR-135a** | 8.30E-03 | 2.80 | 3p21.1/12q23 | - |
| 55 | miR-34a | 8.42E-03 | 2.58 | 1p36.22 | - |
| 56 | miR-28 | 8.72E-03 | 1.94 | 3q28 | - |
| 57 | miR-412 | 9.41E-03 | 2.12 | 14q32.2 | 323-656 |
| 58 | miR-877 | 9.84E-03 | 1.91 | 6p21.33 | - |
| 59 | miR-628-5p | 1.04E-02 | 2.09 | 15q21.3 | - |
| 60 | miR-532-3p | 1.06E-02 | 1.95 | 19q13.41 | - |
| 61 | miR-625 | 1.15E-02 | 2.11 | 14q23.3 | - |
| 62 | miR-34b | 1.19E-02 | 2.07 | 11q33.1 | 34b-34c |
| 63 | miR-31 | 1.28E-02 | 2.62 | 9p21.3 | - |
| 64 | **miR-181a** | 1.46E-02 | 2.05 | 1q32.1/9q33.3 | 181a-181b |
| 65 | **miR-32** | 1.46E-02 | 2.80 | 9q31.3 | - |
| 66 | **miR-106b** | 1.51E-02 | 2.48 | 7q22.1 | 106b-25 |
| 67 | **miR-146a** | 1.52E-02 | 2.14 | 5q33.3 | - |
| 68 | miR-210 | 1.52E-02 | 2.87 | 11p15.5 | - |
| 69 | miR-499-5p | 1.54E-02 | 6.60 | 20q11.2 | - |
| 70 | miR-140 | 1.58E-02 | 2.35 | 16q22.1 | - |
| 71 | miR-188 | 1.68E-02 | 1.99 | Xp11.23 | 532-660 |
| 72 | miR-610 | 1.68E-02 | 2.54 | 11p14.1 | - |
| 73 | **miR-27a** | 1.70E-02 | 2.45 | 19p13.12 | 24-23a |
| 74 | miR-142-5p | 1.70E-02 | 2.47 | 17q22 | - |
| 75 | miR-603 | 1.75E-02 | 1.80 | 10p12.2 | - |
| 76 | miR-660 | 1.75E-02 | 2.13 | Xp11.23 | 532-502 |
| 77 | miR-19a | 1.75E-02 | 2.79 | 13q31.3/Xq26.2 | 17-92/106a-363 |
| 78 | miR-649 | 1.78E-02 | 1.91 | 22q11.21 | - |
| 79 | miR-140-3p | 1.98E-02 | 2.12 | 16q22.1 | - |
| 80 | miR-300 | 2.00E-02 | 2.30 | 14q32.31 | 543-655 |
| 81 | **miR-335** | 2.11E-02 | 2.11 | 7q32.2 | - |
| 82 | miR-206 | 2.20E-02 | 1.84 | 6p12.2 | 206-133b |
| 83 | miR-20b | 2.20E-02 | 2.18 | 13q31.3/Xq26.2 | 17-92/106a-363 |
| 84 | miR-130b | 2.25E-02 | 2.19 | 22q11.21 | 301b-130b |
| 85 | miR-183 | 2.40E-02 | 2.50 | 7q32.2 | 183-182 |
| 86 | miR-652 | 2.44E-02 | 2.36 | Xq22.3 | - |
| 87 | miR-133b | 2.44E-02 | 2.41 | 6p12.2 | 133b-206 |
| 88 | **miR-191*** | 2.56E-02 | 1.97 | 3p21.31 | 425-191 |
| 89 | **miR-19b** | 2.58E-02 | 2.62 | 13q31.3/Xq26.2 | 17-92/106a-363 |
| 90 | miR-212 | 2.71E-02 | 1.97 | 17p13.3 | 212-132 |
| 91 | miR-194 | 2.73E-02 | 2.09 | 1q41/11q13.1 | 215-194/192-194 |
| 92 | **miR-100** | 2.82E-02 | 2.82 | 11q24.1 | 100-let7a |
| 93 | miR-1234 | 2.88E-02 | 1.91 | 8q24.3 | - |
| 94 | miR-182 | 2.96E-02 | 2.29 | 7q32.2 | 183-182 |
| 95 | miR-888 | 3.28E-02 | 2.15 | 5q33.1 | - |
| 96 | **miR-30e-5p** | 3.55E-02 | 2.27 | 1p34.2 | 30e-30c |
| 97 | miR-574 | 3.60E-02 | 2.16 | 4p14 | - |
| 98 | miR-135b | 3.60E-02 | 2.31 | 1q32.1 | - |
| 99 | **miR-125b** | 3.60E-02 | 2.43 | 11q24.1/21q21 | - |
| 100 | miR-502 | 3.77E-02 | 1.79 | Xp11.23 | 500-502 |
| 101 | miR-320 | 3.77E-02 | 1.74 | 8p21.3 | - |

**Table 1 MicroRNAs differentially expressed (*P* ?<? 0.05) between MM (n = 33) and controls (n = 9) depicting chromosomal location and where relevant their expression within a microRNA cluster.** *(Continued)*

| 102 | miR-421 | 3.77E-02 | 1.76 | Xq13.2 | 421-374b |
|---|---|---|---|---|---|
| 103 | miR-129-3p | 3.77E-02 | 1.80 | 7q32.1/11p11.2 | - |
| 104 | **miR-190b** | 4.03E-02 | 2.02 | 1q21.3 | - |
| 105 | miR-18a | 4.44E-02 | 2.21 | 13q31.3/Xq26.2 | 17-92/106a-363 |
| 106 | miR-549 | 4.44E-02 | 2.25 | 15q25.1 | - |
| 107 | **miR-338-5p** | 4.45E-02 | 1.97 | 17q25.3 | 338-657 |
| 108 | miR-576-3p | 4.60E-02 | 4.80 | 4q25 | - |
| 109 | miR-133a | 4.62E-02 | 2.32 | 6p12.2 | 133b-206 |
| -1 | **miR-373*** | 1.39E-03 | -1.82 | 19q13.42 | 371-373 |
| -2 | miR-378* | 2.43E-03 | -3.34 | 5q33.1 | - |
| -3 | miR-143 | 8.83E-03 | -1.80 | 5q33.1 | - |
| -4 | **miR-15a** | 1.51E-02 | -1.67 | 13q14 | 15a-16 |
| -5 | miR-337 | 1.52E-02 | -1.65 | 14q32.2 | 493-432 |
| -6 | **miR-223** | 1.54E-02 | -3.82 | Xq12 | - |
| -7 | miR-369-3p | 1.55E-02 | -1.83 | 14q32.31 | 323-656 |
| -8 | miR-520g | 1.58E-02 | -1.90 | 19q13.42 | 517-518 |
| -9 | miR-485-5p | 1.75E-02 | -3.18 | 19q13.42 | 517-518 |
| -10 | miR-524* | 1.86E-02 | -1.83 | 19q13.42 | 517-518 |
| -11 | miR-520h | 2.20E-02 | -1.98 | 19q13.42 | 517-518 |
| -12 | miR-516-3p | 2.20E-02 | -1.81 | 19q13.42 | 517-518 |
| -13 | miR-519d | 2.20E-02 | -1.78 | 19q13.42 | 517-518 |
| -14 | miR-371-3p | 2.34E-02 | -3.58 | 19q13.42 | 517-518 |
| -15 | miR-455 | 2.88E-02 | -1.88 | 9q32 | - |
| -16 | miR-520b | 2.96E-02 | -1.85 | 19q13.42 | 517-518 |
| -17 | miR-518d | 3.28E-02 | -2.09 | 19q13.42 | 517-518 |
| -18 | miR-624 | 3.28E-02 | -1.56 | 14q12 | - |
| -19 | miR-296 | 3.59E-02 | -1.85 | 21q22.12 | - |
| -20 | **miR-16** | 4.69E-02 | -1.83 | 13q14 | 15a-16 |

Positive values are up-regulated in MM and negative values down-regulated. MicroRNAs in common with Zhou *et al* [11] are denoted in red font and Pichiorri *et al* [12] in bold font.

aberrantly expressed in MGUS samples (*P* < 0.05; Additional file 1 Table S3); 28 microRNAs were up-regulated and 11 down-regulated. Nine of these were consistent with a previous study [12], including up-regulation of *miR-21*, the most up-regulated microRNA identified (10.3 fold change; Figure 2A), *miR-222* and *miR-342* (Figure 2E, H respectively), and down-regulation of *miR-200b* (Figure 2F). Expression levels of MGUS-associated microRNAs distinguished between MGUS and controls by cluster analysis (Additional file 1 Figure S2), and correctly predicted 12/13 (92%) the presence of this disorder by SVM analysis.

## Different genetic subtypes of MM have distinct microRNA expression profiles

Recently it has become increasingly clear that MM displays an enormous genomic complexity that underlies the clinical heterogeneity observed with this disease. The presence of recurrent chromosomal abnormalities such as IgH translocations and del(13q) have been shown to have prognostic significance in multiple studies and as a consequence cytogenetic classification is increasingly being used to stratify MM patients in clinical practice although not yet routinely [15,16]. In order to investigate microRNA expression in the different genetic subtypes of MM, twenty-six cases (for which there was sufficient material) were cytogenetically classified using the micro-FISH technique [17]. Individual patient karyotype data are summarized in Additional file 1 Table S1. Fourteen of 26 (54%) of MM cases harbored an IgH translocation and 7/26 (27%) exhibited the 13q (*RB*) deletion. Of the translocation group eight (57%) displayed the t(11;14) translocation, three (21%) cases the t(4;14) translocation, and three cases had an IgH translocation with an un-known (tested) fusion partner. Ten (38%) MM patients had no detectable chromosomal abnormality using our FISH probe sets, although it is probable that these cases had undetected abnormalities.

The majority of MM tumors harbor IgH translocations, the most common fusion partners being 11q13
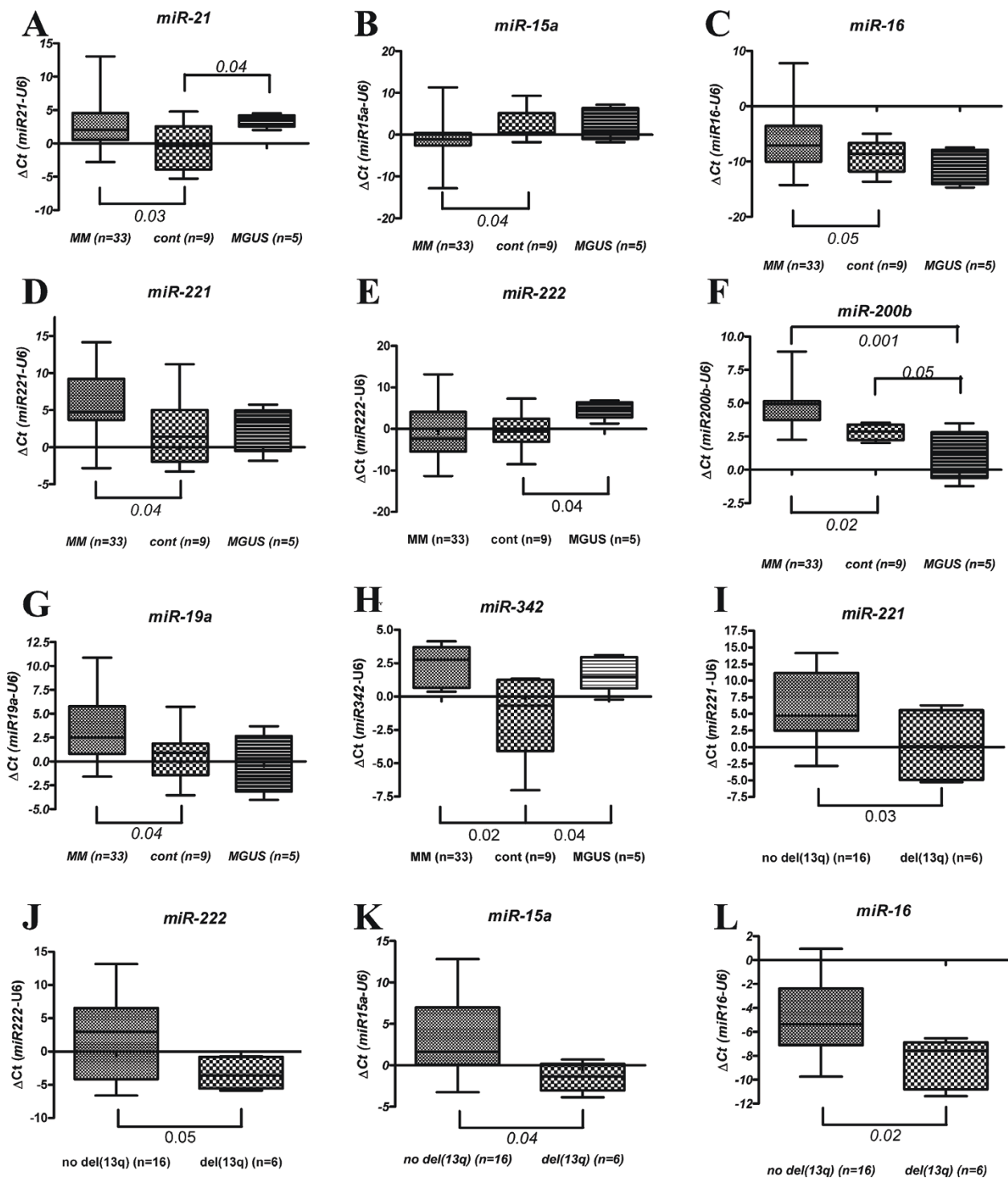
**Figure 2 Validation of microarray data by qRT-PCR**. Expression levels of (A) *miR-21*, (B) *miR-15a*, (C) *miR-16*, (D) *miR-221*, (E) *miR-222*, (F) *miR-200b*, (G) *miR-19a* and (H) *miR-342* in MM (n = 33), control (n = 9) and MGUS (n = 5) samples measured by qRT-PCR. Expression levels of (I) *miR-221*, (J) *miR-222*, (K) *miR-15a* and (L) *miR-16* in MM patients with and without del(13q). P values were calculated by Mann-Whitney independent *t*-test.

(*CCND1*) and 4p16 (*FGFR3*) [18]. A comparison between cases containing an IgH translocation with those that did not identified seven differentially expressed (P < 0.05) microRNAs (Table 2). Expression levels were distinct in MM cases containing the IgH translocation (Figure 3A), and predicted 24/26 (92%) of cases by SVM analysis. Seventeen microRNAs were identified as being associated with the most common IgH translocation event, t(11;14) (*IgH:CCND1*) (P < 0.05; Table 2), four of which (*miR-375, miR-650, miR-193a* and *miR-582*) were consistent with other studies [19,20]. Expression levels of these microRNAs distinguished cases with t(11;14) (Figure 3B), and correctly predicted the presence of this translocation in 24/26 (92%) of cases by SVM analysis. Three cases in our cohort had the t(4;14) (*IgH:FGFR3*) translocation. Eight microRNAs were associated with this translocation (P < 0.05; Table 2), three of which (*miR-99b, miR-342* and *miR-214*) were previously identified [19,20]. Expression levels of these microRNAs distinguished cases with the t(4;14) translocation (Figure 3C) and correctly predicted all cases containing this translocation. Interestingly, four of the six up-regulated microRNAs are encoded on either chromosome 4 or 14 (*miR-376a* (14q33.21), *miR-342* (14q32.2), *miR-574* (4p14) and *miR-577* (4q26)), and of the two microRNAs (*miR-520c* and *miR-376a*) aberrantly expressed in both t(4;14) and t(11;14) cases, the latter is encoded proximal to the IgH breakpoint region at 14q32.

A comparison between microRNA expression in del (13q) MM patients with patients not harboring this deletion identified 28 microRNAs by ANOVA (P < 0.05; Table 2). Expression levels of these microRNAs distinguished cases that were del(13q) by cluster analysis (Figure 3D), and correctly predicted its presence in 22/26 (85%) of cases by SVM analysis. Eight of these microRNAs were consistent with research by Gutiérrez and colleagues [19], including down-regulation of *miR-15a* and *miR-16*, encoded within the 13q14 locus (Figure 2K and 2L respectively).

### MicroRNA expression is associated with clinical parameters and survival

The most common paraprotein isotypes associated with MM are IgG (60%) and IgA (24%) [21]. It has been reported that IgA myeloma has a poorer prognostic outcome than patients with IgG myeloma [22], although it should be noted that we (data not shown) and others [23] did not find any survival differences between isotypes. Consequently we compared expression levels between thirteen cases of IgG-type MM with eight cases of IgA-type. This resulted in the identification of twenty-one differentially expressed microRNAs (P < 0.05; Additional file 1 Table S5). Expression levels of these microRNAs were distinct between IgA and IgG MM cases (Additional file 1 Figure S4), and correctly predicted isotype for 20/21 (95%) of cases by SVM analysis.

Approximately 10% of MM patients secrete only light chains instead of the complete immunoglobulin molecule [21]. So called "light chain only myeloma" or "Bence Jones myeloma" had been reported to have a poorer prognostic outcome [22,24] although a more recent (and comprehensive) study found no such difference [23]. In our cohort eight MM cases had light chain only myeloma. Twenty-seven microRNAs were identified as being differentially expressed compared to non-light chain only MM cases (Additional file 1 Table S6). Expression levels of these microRNAs correctly predicted light-chain only myeloma in all cases.

As we had successfully identified microRNAs associated with clinical outcome in diffuse large B-cell lymphoma [7], we assessed differences in microRNA expression between poor outcome and good outcome in MM cases, using event-free survival (EFS) as our clinical criterion (median follow-up = 20 months). Samples from patients with event-free survival (n = 21) were contrasted with those from patients who had relapsed or died in this interval (n = 7). Thirty-two microRNAs were identified as being differentially expressed (P < 0.05) (Additional file 1 Table S7). These microRNAs correctly predicted relapse in 24/28 (86%) of cases by SVM analysis. To investigate the effect of individual microRNA expression levels on clinical outcome we carried out log-rank (univariate) survival analysis and found that low expression of *miR-153, miR-490, miR-455, miR-642, miR-500*, and *miR-296*, and high expression of *miR-548d, miR-373, miR-554* and *miR-888* were associated (P < 0.05) with EFS (Figure 4).

### Discussion

In this study we used microarray technology to elucidate the complete miRNome (miRBase version 13.0) of purified PCs from 33 MM and 5 MGUS patients and compared their expression with counterpart normal PCs. One hundred and twenty-nine microRNAs were identified as being aberrantly expressed in MM. Thirty-nine (30%) of these were previously reported to be aberrantly expressed in MM by Pichiorri *et al* [12] and/or Zhou *et al* [11]. The reason for these discrepancies probably reflects the use of differing statistical, microarray and sampling methods. Of note the former study only examined 10 MM and 4 controls, whilst the latter study included only 2 controls for analysis. Consistent with the findings of Pichiorri and colleagues we found that *miR-21* was up-regulated in both MM and MGUS as were all seven microRNAs encoded by *miR-17-92* cluster (average fold-change = 2.1; range 1.75-2.78).

**Table 2 MicroRNAs associated (*P* < 0.05) with different genetic subtypes depicting chromosomal location.**

| Cytogenetic group | microRNA | Fold change | *P*-value | Chromosome |
|---|---|---|---|---|
| IgH trans (n = 14) | miR-590 | 3.28 | 1.62E-02 | 7q11.23 |
| | miR-886 | 1.63 | 1.46E-02 | 5q31.1 |
| | miR-33b | 1.48 | 2.46E-02 | 17p11.2 |
| | miR-184 | -1.43 | 3.87E-02 | 15q25.1 |
| | miR-139 | -1.44 | 1.47E-02 | 11q13.4 |
| | miR-508 | -1.63 | 4.55E-03 | Xq27.3 |
| | miR-579 | -1.66 | 2.43E-02 | 5p13.3 |
| t(11;14) (n = 8) | miR-202* | 2.16 | 3.10E-03 | 10q26.3 |
| | miR-520c | 2.05 | 1.26E-03 | 19q13.42 |
| | miR-890 | 2.04 | 4.39E-02 | Xq27.3 |
| | **miR-582** | 1.91 | 4.06E-02 | 5q12.1 |
| | miR-122a | 1.88 | 1.40E-02 | 3p21.31 |
| | miR-526b* | 1.86 | 1.09E-04 | 19q13.42 |
| | **miR-375** | -1.74 | 2.04E-02 | 2q35 |
| | miR-543 | -1.59 | 3.46E-02 | 14q33.21 |
| | **miR-650** | -1.58 | 4.00E-02 | 22q11.22 |
| | **miR-193a** | -1.51 | 1.35E-02 | 17q11.2 |
| | miR-147b | -1.46 | 2.76E-02 | 15q21.1 |
| | miR-526a | -1.51 | 4.68E-02 | 19q13.42 |
| | miR-542 | -1.56 | 1.81E-02 | Xq26.3 |
| | miR-301 | -2.22 | 4.80E-02 | 17q22 |
| | miR-26b | -2.62 | 3.81E-02 | 2q35 |
| | miR-376a | -2.63 | 2.68E-02 | 14q33.21 |
| | miR-21 | -2.82 | 5.19E-03 | 17q23.1 |
| t(4;14) (n = 3) | miR-376a | 3.48 | 1.23E-02 | 14q33.21 |
| | miR-574 | 3.11 | 1.23E-02 | 4p14 |
| | **miR-99a** | 2.53 | 1.23E-02 | 21q21.1 |
| | **miR-214** | 2.12 | 2.77E-02 | 1q24.3 |
| | miR-577 | 1.97 | 3.85E-02 | 4q26 |
| | **miR-342** | 1.79 | 3.50E-02 | 14q32.2 |
| | miR-935 | -2.07 | 3.55E-02 | 19q13.42 |
| | miR-520c | -2.23 | 3.85E-02 | 19q13.42 |
| 13q(del) (n = 6) | **miR-221** | -9.71 | 1.18E-04 | Xp11.3 |
| | miR-222 | -9.50 | 1.78E-03 | Xp11.3 |
| | **let-7b** | -5.15 | 3.38E-03 | 22q13.31 |
| | let-7a | -4.92 | 3.72E-03 | 9q22.32/11q24.1 |
| | **let-7c** | -4.86 | 6.70E-03 | 21q21.1 |
| | **miR-15a** | -4.42 | 6.39E-03 | 13q14 |
| | **miR-20a** | -4.41 | 1.47E-03 | 13q31 |
| | miR-107 | -4.40 | 3.23E-03 | 10q23.31 |
| | miR-26a | -4.37 | 3.26E-03 | 3p22.2/12q14.1 |
| | miR-103 | -4.30 | 8.98E-03 | 5q34/20p13 |
| | miR-142 | -4.13 | 1.15E-03 | 17q22 |
| | miR-195 | -3.97 | 6.98E-03 | 17p13.1 |
| | miR-146a | -3.92 | 1.46E-03 | 5q33.3 |
| | miR-23a | -3.80 | 6.44E-03 | 19p13.12 |
| | miR-92a | -3.77 | 3.57E-03 | 13q31 |
| | miR-27a | -3.58 | 2.25E-03 | 19p13.12 |
| | miR-361 | -3.46 | 4.19E-03 | Xq21.2 |
| | miR-145 | -3.43 | 7.10E-03 | 5q32 |
| | miR-92b | -3.37 | 9.82E-03 | 13q31 |

**Table 2 MicroRNAs associated (*P* < 0.05) with different genetic subtypes depicting chromosomal location.** *(Continued)*

| | | | |
|---|---|---|---|
| **miR-19a** | -3.36 | 5.57E-03 | 13q31 |
| miR-199a* | -2.93 | 5.93E-03 | 19p13.2/1q24.3 |
| miR-34a | -2.89 | 2.56E-03 | 1p36.22 |
| miR-188 | -2.79 | 8.43E-03 | Xp11.23 |
| **miR-196a** | -2.40 | 5.60E-03 | 17q21.32 |
| miR-485 | -2.33 | 4.18E-03 | 19q13.42 |
| **miR-16** | -2.23 | 7.79E-03 | 13q14 |
| miR-493 | -1.89 | 2.46E-03 | 14q32.2 |

Comparison between MM cases with (positive fold change) and without (negative fold change) chromosomal abnormality as indicated in text. MicroRNAs also identified by Gutiérrez *et al* [19] or Lionetti *et al* [20] are depicted in bold type.

However unlike that study, although *miR-32* and *miR-181a* were identified as being up-regulated by microarray analysis, we failed to find any significant difference in expression levels by qRT-PCR (data not shown). Surprisingly 10 of the 20 down-regulated microRNAs identified in the current study are encoded by the *miR-371-1323* cluster located at 19q13.42. This cluster consists of 49 microRNAs, 39 of which were present on our microarray. All members of this cluster were down-regulated (average 1.6-fold; range 1.1-2.5). Interestingly, members of this cluster (*miR-373* and *miR-520c*) have been demonstrated to be associated with tumor invasion and metastasis [25].

The vast majority (109/129 (85%)) of microRNA aberrantly expressed in MM were up-regulated, consistent with the findings of Zhou *et al* [11]. We [7,26] (and others[27]) found the same pattern in other B-cell malignancies. In contrast, we [10] (and others [28]) recently demonstrated that T-cell malignancies are associated with a global decrease in microRNA expression. This suggests a fundamental difference in microRNA biogenesis between B and T-cell malignancies. A possible explanation for this distinction comes from our previous observation that components of the microRNA biosynthetic pathway (i.e. the microprocessor complex (*DGCR8* and *Drosha*)) are up-regulated in B-cell malignancies (including MM) but down-regulated in T-cell malignancies [29].

Using the same array as in this study, we previously identified 60 and 119 microRNAs aberrantly expressed in diffuse large B-cell lymphoma (DLBCL) [7] and Sézary syndrome (SzS) (T-cell lymphoma) respectively [10]. A comparison with MM-associated microRNAs revealed that 25/60 (42%) and 47/119 (40%) of microRNAs
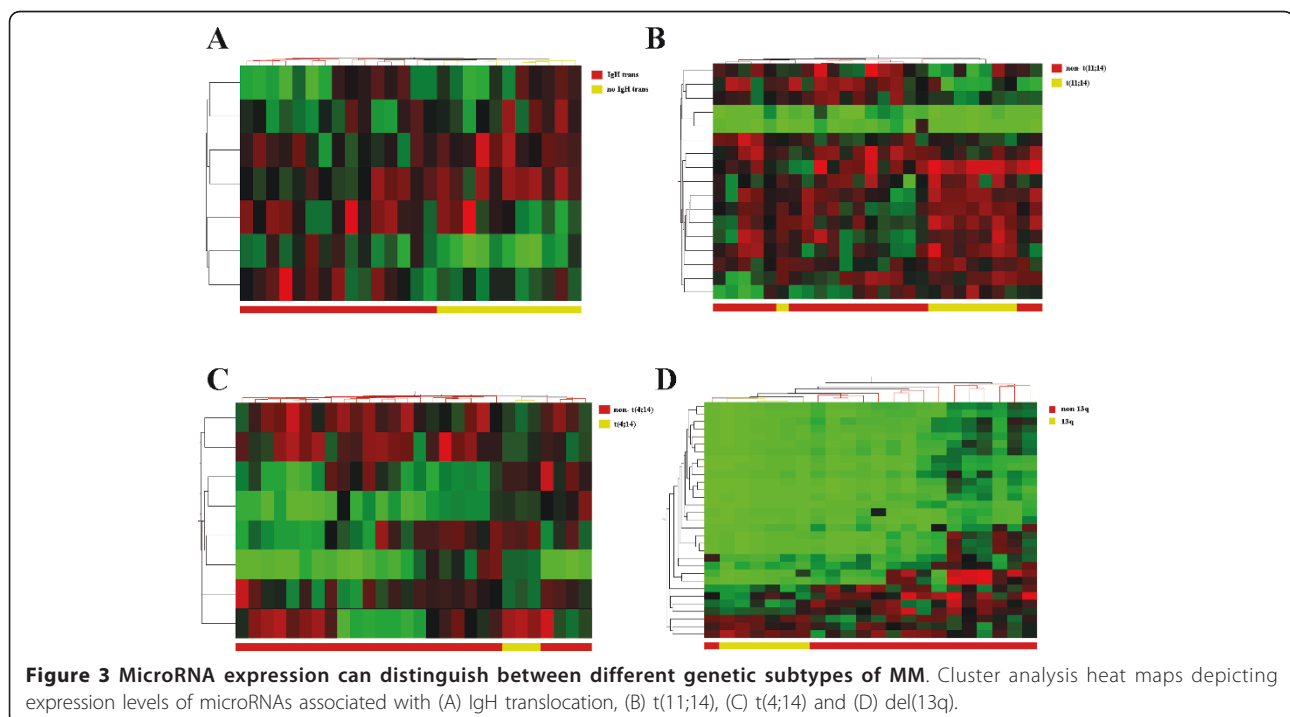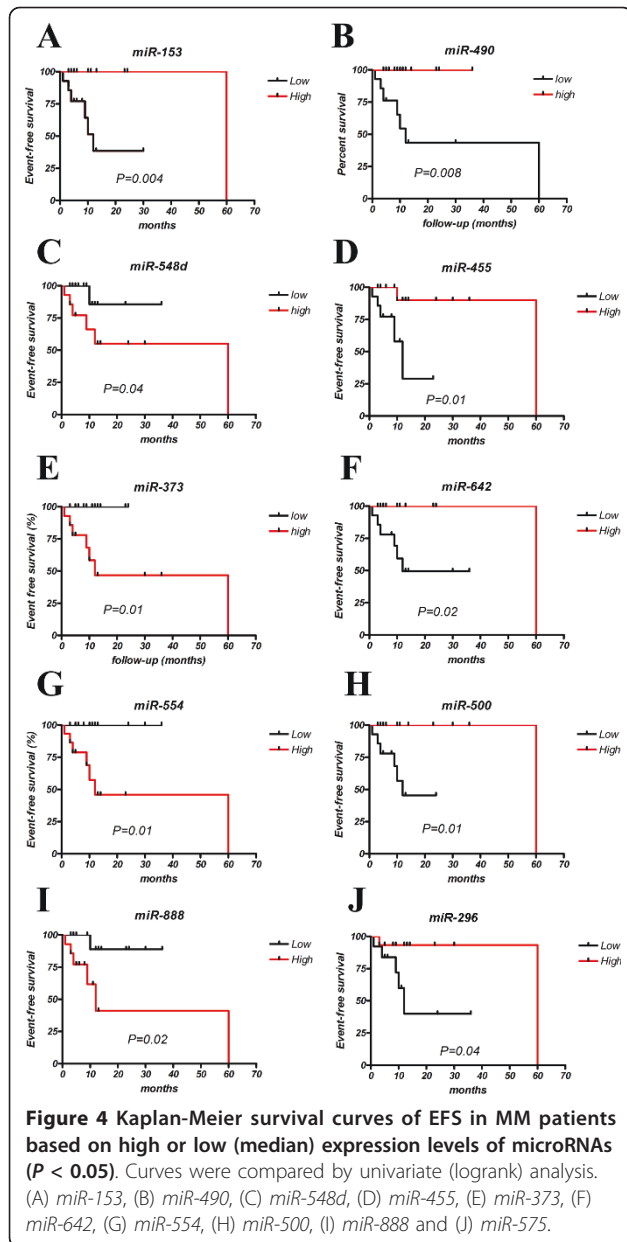


**Figure 3 MicroRNA expression can distinguish between different genetic subtypes of MM**. Cluster analysis heat maps depicting expression levels of microRNAs associated with (A) IgH translocation, (B) t(11;14), (C) t(4;14) and (D) del(13q).

**Figure 4 Kaplan-Meier survival curves of EFS in MM patients based on high or low (median) expression levels of microRNAs (*P* < 0.05)**. Curves were compared by univariate (logrank) analysis. (A) *miR-153*, (B) *miR-490*, (C) *miR-548d*, (D) *miR-455*, (E) *miR-373*, (F) *miR-642*, (G) *miR-554*, (H) *miR-500*, (I) *miR-888* and (J) *miR-575*.



| | Fold change | | | |
|---|---|---|---|---|
| | **MM** | **DL** | **SzS** | **Chromosome** |
| miR-100 | 2.82↑ | 7.33↑ | -4.79↓ | 11q24.1 |
| miR-125b | 2.43↑ | 24.18↑ | -6.85↓ | 11q24.1/21q21 |
| miR-128a | 2.82↑ | -2.12↓ | -3.09↓ | 2p21.3/3p22.3 |
| miR-128b | 2.25↑ | -2.37↓ | -3.82↓ | 2p21.3/3p22.3 |
| miR-133b | 2.41↑ | 2.03↑ | -3.34↓ | 6p12.2 |
| miR-143 | -4.79↓ | -4.48↓ | -6.85↓ | 5q33.1 |
| miR-181a | 2.05↑ | -2.81↓ | -4.40↓ | 1q32.1/9q33.3 |
| miR-223 | -3.82↓ | -2.67↓ | -13.99↓ | Xq12 |
| miR-335 | 2.11↑ | -2.42↓ | -4.11↓ | 7q32.2 |
| miR-361 | 12.66↑ | -2.87↓ | -13.00↓ | Xq21.2 |
| miR-455 | 2.25↑ | 2.05↑ | -3.14↓ | 9q32 |
| miR-574 | 2.16↑ | 3.46↑ | 12.82↑ | 4p14 |
| miR-652 | 2.36↑ | -2.11↓ | -3.14↓ | Xq22.3 |
| miR-99a | 3.47↑ | 6.13↑ | -4.48↓ | 21q21 |

**Figure 5 MicroRNAs commonly dysfunctionally expressed in multiple lymphoma types**. (A) Venn diagram showing relationship between microRNAs aberrantly expressed in diffuse large B-cell lymphoma (DLBCL) [7], Sezary syndrome (SzS) [10] and multiple myeloma (MM) ((Table 1) (Additional file 1, Table S2)). (B) Tables of microRNAs aberrantly expressed in all lymphoma types depicting fold change relative to normal counterpart controls.

aberrantly expressed in DLBCL and SzS respectively were also aberrantly expressed in MM (Figure 5 andAdditional file 1 Table S2). Fourteen microRNAs were dysfunctionally expressed in all three lymphoma types with *miR-223* and *miR-143* commonly down-regulated and *miR-574* commonly up-regulated. Although this analysis is by no means exhaustive this suggests the presence of a common lymphoma microRNA signature. Down-regulation of *miR-223* also has been demonstrated in hepatocellular carcinoma [30], acute myeloid leukemia [31] and chronic lymphocytic leukemia [32,33], and shown to regulate the important hematopoietic regulator *LMO2* [34] that is commonly expressed in lymphoma
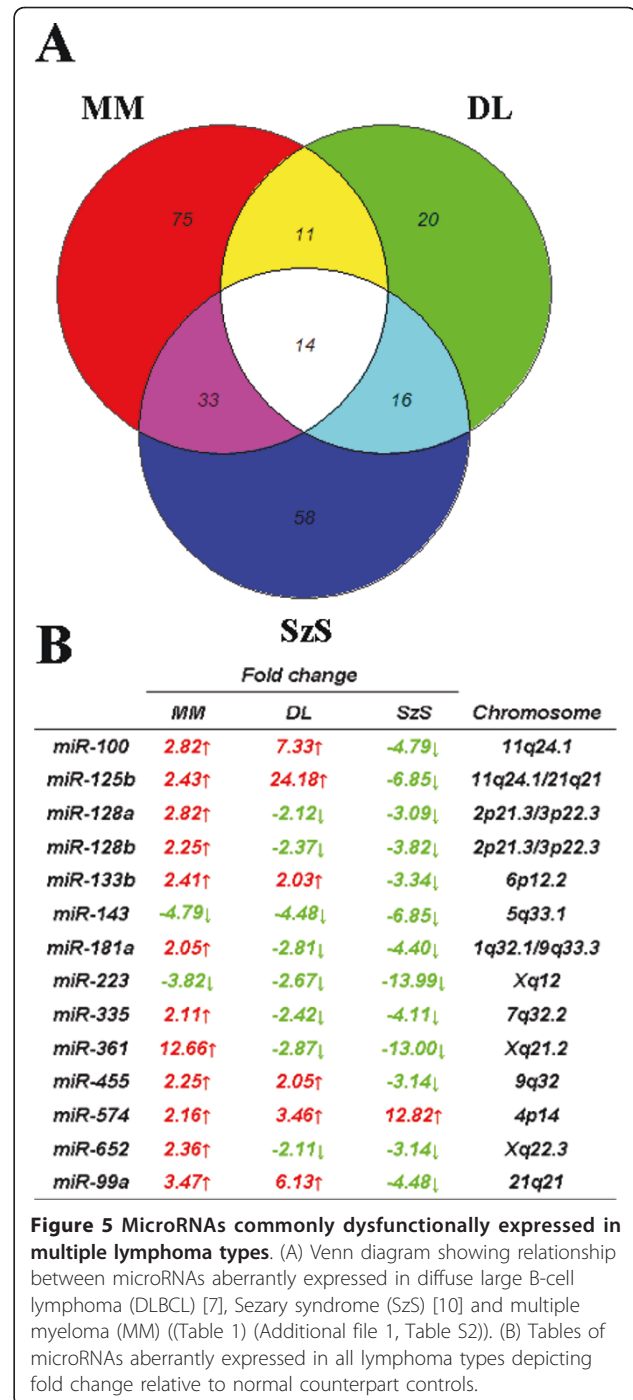
[35]. Similarly, *miR-143* has been reported to be commonly down-regulated in hematological malignancy [36]. We are currently investigating expression of these microRNAs in a range of lymphoma types to see if their dysregulation is truly a common feature of lymphoma.

MGUS has been shown to consistently proceed the development of MM [37] and as such is considered to represent a pre-malignant model for MM pathogenesis.

Twenty-three of the 39 (59%) microRNAs aberrantly expressed in MGUS were also aberrantly expressed in MM (Additional file 1 Table S3) suggesting common microRNA expression events in MM progression. Thirty-seven microRNAs were identified as being differentially expressed between MGUS and MM (Additional file 1 Table S4), only 9 of these were also differentially expressed in MGUS compared to controls (i.e. specific for MGUS) including *miR-21* as previously identified [12]. The relationship between microRNAs dysfunctionally expressed in MGUS and MM are shown in Additional file 1 Figure S3. *miR-21* over-expression has been linked to an anti-apoptotic phenotype and enhanced tumor growth [38,39] and as such may represent an important aberration in the early pathogenesis of MM.

Next we investigated the identity of microRNAs associated with the major genetic subtypes of MM. Two previous studies by Lionetti *et al* [20] and Gutiérrez *et al* [19] have also examined microRNA expression associated with recurrent chromosomal abnormalities in MM. Generally our findings were consistent with these studies although microRNAs associated with specific abnormalities did not always completely overlap. The cause for discrepancies between studies, including lack of concordance between the two previously published studies, again probably represents differences between microarray platforms, statistical techniques and samples used for analysis. For example the study of Gutiérrez *et al* considered cases in which del(13q) was the sole chromosomal abnormality and deregulated microRNAs were identified on the basis of their expression relative to control PCs, whereas our study and that of Lionetti *et al* considered the difference between MM cases with and without specific chromosomal abnormalities [19,20]. The latter study considered genetic abnormalities in the context of a gene-expression defined molecular translocation/cyclin (TC) classification system [20]. Such differences between studies are a reflection of the relative immaturity of the microRNA field that has only formally existed for the last 9 years, and is something that will surely be resolved in future years.

Interestingly, from the 28 microRNAs associated with the presence of del(13q), all seven microRNAs encoded by the *miR-17-92* cluster (encoded at 13q31) were down-regulated (average 3.1 fold; range 1.2-4.1). Recent data suggests that unlike the characteristic del(13q) karyotype observed in chronic lymphocytic leukemia (CLL) defined by loss of the 13q14 locus, the minimal deleted region of 13q loss in MM extends from 13q14 to the 13q34 locus [40], consistent with down-regulation of the *miR-17-92* cluster observed in these cases. Although down-regulation of the *miR-17-92* cluster was also reported by Gutiérrez *et al* [19], this finding is counter-intuitive as members of this cluster are widely believed to function as oncogenes [41] whilst the presence of del (13q) is associated with an adverse prognostic outcome in MM [42]. A possible explanation for this apparent paradox is that although widely up-regulated in a wide range of cancers [9,26,43], the *miR-17-92* cluster has been proposed to act as both tumor-suppressor or oncogene depending upon the cellular context [44-46]. Recently we reported that this cluster is down-regulated in T-cell lymphoma where it was shown to have tumor-suppressor properties [10]. Consequently, it is possible that the cluster is similarly acting as a tumor-suppressor in del(13q) MM cases although it should be noted that up-regulation of this cluster is associated with MM diagnosis (Table 1) and at least one member of this cluster (*miR-19*) was found to antagonize MM tumor development in a murine model [12].

We also report for the first time a correlation between microRNA expression and isotype in MM including the presence of light-chain only myeloma. Such information might be useful in providing an alternative clinical classification of new MM patients. Finally, we examined how the expression levels of individual microRNAs might influence clinical outcome of MM patients by correlating expression values with adverse effects using Kaplan-Meier survival analysis. We found that high expression of *miR-153, miR-490, miR-455, miR-642, miR-500*, and *miR-296* were associated with better EFS in our cohort of patients whilst the opposite was true for *miR-548d, miR-373, miR-554* and *miR-888*. Interestingly, the most discriminatory of these microRNAs (i.e. *miR-153*) has been shown to target *BCL2* and *MCL1* [47] and that over-expression of both of these are associated with poor prognostic outcome in MM [48]. Low expression of *miR-455* has also been associated poor overall survival in endometrial serous adenocarcinoma [49], and low levels of *miR-296* shown to be associated with increased prostate tumor growth and invasion properties via targeting of *HMGA* [50]. It should be noted however that these data need to be tested in an independent test cohort before any firm conclusions can be drawn about the prognostic ability of these microRNAs in MM patient survival, whilst the current results provide the experimental basis for future validation experiments.

## Conclusions

In summary, these results indicate that aberrant expression of microRNAs is a common feature of MM as well as pre-malignant MGUS, and that this phenomenon is associated with genetic and clinical subtype as well as clinical outcome. This data reinforces the complex nature of MM and hopefully will provide useful information in clarifying the molecular basis of this disease in future studies.

## Methods

### Patient samples and cell lines

Bone marrow samples were obtained from patients attending the Department of Haematology, John Radcliffe Hospital, Oxford, UK or Dubrava University hospital Zagreb, Croatia. Patients were diagnosed as MM (n = 33) or MGUS (n = 5) according to criteria described in the myeloma management guidelines [51]. The median age of patients was 67 yrs (range 43-89 yrs). Individual patient details are shown in Additional file 1 Table S1. Plasma cells (PC) were purified from bone marrow aspirates by positive immunomagnetic selection (CD138^{+}) according to manufacturer's instructions (Miltenyi Biotec, Bisley, UK). PCs obtained from the bone marrow of nine healthy individuals were used as controls. The purity of PC samples was > 90% CD138^{+} as measured by immunohistochemical staining.

Patient samples were collected in accordance with the Declaration of Helsinki and approved by the local ethics committee (Oxforshire Regional Ethics Committee; Ref no. 07/Q1606/25). Patients gave written informed consent for the sample collection.

MM cell lines, NCI-H929, JJN3, RPMI8226 and Thiel were routinely cultured in RPMI 1640 containing 10% fetal calf serum (Invitrogen, Paisley, UK). All cell lines were obtained from the DSMZ cell collection (Braunschweig, Germany) except Thiel which was a kind gift from Prof. Diehl (University of Cologne, Germany).

### Cytogenetic classification of myeloma samples

Purified MM patient PCs ($\sim 10^5$ cells) were classified according to cytogenetic criteria using the micro-FISH technique as previously described [17]. The following probe sets obtained from Vysis (Abborsh Diagnostics, Maidenhead, UK) were used for classification; D13S319 Spectrum-Orange/LSI-13q34-Spectrum-Green (del(13q) probe), IgH Spectrum-Green/FGFR3 Spectrum-Orange (t(4;14) fusion probe), IgH Spectrum-Green/CCND1 Spectrum-Orange (t(11;14) fusion probe), and IgH dual-color (Spectrum-Green/Spectrum-Orange) break-apart probe [52]. At least 100 nuclei were scored for each probe. A 20% cut-off was used for numerical abnormalities and 10% for fusions and break-apart probes as recommended by the European Myeloma Network FISH workshop guidelines http://www.myeloma-europe.org.

### RNA purification and microarray analysis

MicroRNA was isolated from samples ($\sim 10^6$ cells) using Trizol (Invitrogen, Paisley, UK) and RNeasy columns as described by the manufacturer (Qiagen, Crawley, UK). MicroRNA ($\sim$500 ng) were labeled and hybridized to μRNA microarrays as previously described [26] using tonsillar material (pooled from twelve healthy individuals) as a common reference in a dye-balanced design. The arrays contained 655 human probes (miRBase v.10.1). Probe details can be found at http://www.micro-RNAworld.com.

Image analysis was carried out with BlueFuse software (BlueGnome, Cambridge, UK). Raw image data were global loess-normalized within arrays and normalized between arrays using the LIMMA package [53]. The normalized log ratios (average of four replicates per probe) were used for subsequent analysis in Genespring 7.2 (Agilent Technologies, CA, US). ANOVA analysis was used to identify microRNAs differentially expressed between sample types and *P* values were adjusted using the Benjamini-Hogberg correction method. Differentially expressed genes were tested for their ability to predict sample class using the leave-one-out cross-validation support vector machine (SVM) function in Genespring (Polynomial Dot Product (Order 1) Kernel Function. Diagonal Scaling Factor: 0). All microarray data was MIAME compliant and raw data has been deposited in the GEO database (Accession series # GSE243371).

### Quantitative RT-PCR (qRT-PCR)

MicroRNA qRT-PCR was carried out using Taqman probes as described by the manufacturer (Applied Biosystems, Warrington, UK) using 20 ng of microRNA per reaction in a Roche LightCycler 480 machine. Triplicate samples were used throughout. The mean Ct value of each triplicate was used for analysis, by the $\Delta C_t$ method ($\Delta C_t$ = mean $C_t$ of microRNA of interest-mean $C_t$ of U6). Expression levels were compared using Mann-Whitney independent *t*-test (Graphpad Prism v.4.0, La Jolla, CA).

### Survival analysis

Kaplan-Meier survival analysis was carried out on event free survival (EFS) times of MM patients as a function of microRNA expression, using the median value as cut-off. EFS was calculated as the time of diagnosis to the date of clinical relapse, death or last contact. Patients who were relapse-free at time of last contact were censored for analysis. Mean follow-up time was 20 months (range 1-60 months). Curves were compared by univariate (logrank) analysis using GraphPad Prism version 4.00 (La Jolla, CA).

## Reviewers' comments

Reviewer #1: Prof. Neil Smalheiser, Department of Psychiatry, University of Illinois, Chicago, IL, US.

### Reviewer's comments

This is a nice paper and I only have relatively minor comments, which can be addressed by page number:

p. 2. It would not hurt to have some discussion of the miR expression in the control group, in terms of the cell type profile, heterogeneity across individuals, etc. If you included that, I missed it.

**Authors' response**

*Whilst the reviewer has an interesting suggestion i.e. to look at variation in miRNA expression across the control group, the main theme of this research was to look at expression in multiple myeloma, therefore the number of controls samples used (n = 9) does not have sufficient power in order to address that specific question. However, our data does suggest that the control samples varied little in their miRNA expression profile as shown by unsupervised cluster analysis (Figure 1).*

p. 2. You speak of altered miR expression in tumor cells as "aberrant" throughout the paper. Yet not every difference is necessarily aberrant. Some changes may be compensatory; some changes may simply reflect the physiological state of the tumor cells without being linked to tumorigenicity per se (e.g., they may differ in O2 tension or pH).

**Authors' response**

*We whole-heartedly agree that not all differentially expressed miRNAs identified in MM tumor cells will be directly linked with their neoplastic nature but instead may reflect indirect effects such as their abnormal microenvironment and/or interaction with non-tumor cells for example. We believe however that as these effects, direct and indirect, cumulatively represent tumor-associated changes in the miRNA expression profile of cells, that the use of the adjective 'aberrant' in this context is an accurate description of the expression behavior of miRNAs that are 'diverging from the normal' (Oxford Concise Dictionary).*

p. 10 and Methods. It is worthwhile to describe the method of correcting p-values for multiple-testing in more detail.

**Authors' response**

*As stated in the methods section p-values were corrected using the Benjamini-Hogberg multiple testing correction algorithm as implemented in the ANOVA function of the Genespring software package. This algorithm is based on false discovery rate error type and further details can be found in the original publication of this method (Benjamini and Hochberg (1995). Journal of the Royal Statistical Society B, 57, 289-300)). As this method is widely used in microarray analyses we believe that a more in-depth and mathematical description of this method is not warranted for this manuscript.*

p. 10 and throughout. The paper has a nice balance between considering miRs as a clinical tool and as an indication of tumor biology. However, you can go still further to analyze in biological terms WHY the pattern of altered miR expression is the way it is, or

(stated a different way) to analyze what dimensions are most important for SVM prediction. For example, do the altered miRs all share the same TFs driving them?

**Authors' response**

*The reviewer has raised a very important point about addressing the reason for aberrant miRNA expression in MM. However, the causal mechanisms behind miRNA dysregulation in any cancer are complex, although we did try to proffer some insight in the context of MM in both results and discussion sections of this manuscript. Possible mechanisms include chromosomal lesions at miRNA-encoding regions and defects in miRNA biosynthetic machinery, both of which were addressed in this manuscript. Further mechanisms include epigenetic regulation, changes in expression of genes such as E2F that can bind to promoter regions of pri-miRNA sequences, as well as any factor that can regulate levels of the protein-encoding genes that encode for >40% of human miRNAs. The problem with identifying genes such as TFs that are potentially targeted by commonly differentially expressed miRNAs, is that currently the predictive algorithms to carry out this type of analysis perform very poorly (Sethupathy, et al (2006) Nat Methods, 3, 881-886). Therefore we believe that the robustness and hence usefulness of such an in silico approach is limited. Consequently we decided to restrict our discussion to those target genes that have been independently experimentally validated.*

p. 13. You mention that dicer and drosha tend to be up-regulated in B-cell malignancies. It would be desirable to have measured these in the SAME samples reported here and see if dicer and/or drosha levels are tightly correlated with miR up-regulation.

**Authors' response**

*This would be the ideal situation, and in the case of the DLBCL case this comparison was carried out (Lawrie et al (2009) Br. J. Haematol., 145, 545-548). Unfortunately there was insufficient material left from the current study to carry out a similar analysis on the MM samples.*

p. 19, Methods. Were control and tumor samples collected during the same period? stored for similar amounts of time? Assayed in parallel? If not, this could create confounds.

**Authors' response**

*Due to the logistics of obtaining bone marrow samples from ~50 patients/controls it would have been extremely difficult to collect samples over the same period- samples were collected over a ~2 year period from two centers (Oxford and Zagreb). Patient/control samples were assayed in parallel and randomized/anonymized to the researchers processing them in order to minimize confounding variables.*

p. 20, Methods. Was U6 expression the same in control and tumor samples? This should be stated explicitly.

**Authors' response**

*U6 levels were measured in controls and tumor in parallel using the same assay system (i.e. Taqman probe qRT-PCR). There was no significant difference in absolute U6 expression levels (i.e. Ct values) between controls and tumor samples.*

p. 29, Table 1. You have mir-200a listed twice with two different values?! Please check this (very long) table.

**Authors' response**

*This was a mistake. Position 25 in* Table 1*should have read miR-200a\* and not miR-200a, this has been corrected.*

p. 35, Figure 3 Legend. Please spell out DL, MM and SZ

**Authors' response**

*This issue has been addressed.*

Reviewer #2: Prof. Yuriy Gusev, Department of Surgery, University of Oklahoma, OK, USA.

This reviewer provided no comments for publication.

Reviewer #3: Unknown reviewer

**Reviewer's comments**

This manuscript includes a description of microarray analysis for the expression of microRNA in purified CD138+ cells from 33 patients with multiple myeloma (MM), 5 patients with monoclonal gammopathy of undetermined significance (MGUS), and 9 healthy volunteers. The authors found distinct microRNA expression profiles from each group. Similar studies from 2 different groups have been reported. However, in one study only 16 samples from patients with MM, 6 from patients with MGUS, and 6 controls were included and in the another study samples from 52 patients but only 2 controls were studied. The manuscript is clear and concise. The data are convincing and the area of investigation is important. This manuscript is a valuable contribution. Specific comments follow:

1. The authors state several times that they have performed a comprehensive study to elucidate the complete miRNome of the CD138+ cells; however, I could not find how "complete" was defined. The manuscript would be improved by including a consideration about the completeness of the analysis performed. How the microarray analysis was defined as a complete analysis of miRNA and citations would enhance the manuscript.

**Authors' response**

*The 'complete miRNome' refers to the microarrays (µRNA microarrays) used for these experiments which contain probes against every known miRNA (the miRNome) as defined by the reference depository for miRNAs (miRBase-* http://www.mirbase.org*) (Griffiths-Jones et al (2006), Nucleic Acids Research, 34, D140-144). At the*

*time when these experiments were started (June 2009), the miRBase version was 13.0 which included 655 human sequences. The authors readily accept that the miRNA database has grown since this time and that the current version of the miRBase miRNome is version 16 and contains 1048 human sequences. This assertion has been modified in the text to read 'complete miRNome (miRBase version 13.0)' in the text in order to avoid confusion.*

2. The "leave-one-out cross-validation support vector machine algorithm" (mentioned on page 7) is not defined or described in the methods section. The support vector machine algorithm is also mentioned in the abstract but the manuscript would be improved by describing and referencing this technique.

**Authors' response**

*The support vector machine (SVM) algorithm is a supervised learning method commonly used for classification in microarray experiments (along with k-nearest neighbor algorithm) and is based on non-probabilistic binary linear classification (Cortes & Vapnik (1995) Machine learning,* **20**). *We believe that as the parameters used for its implementation within the Genespring package as described in the materials and methods section are already given, that this would be sufficient information for others to carry out the same analysis.*

3. In the results the authors state that 8 miRNA were chosen to be measured by qRT-PCR to validate the microarray data. They state, "These data were consistent with the microarray results". In the discussion the authors indicate that "miR-32 and miR-181a were identified as being up-regulated by microarray analysis" by qRT-PCR failed to show any significant differences in expression levels. Were there other miRNA studied by qRT-PCR? Why weren't miR-32 and miR181a included in Figure 2?

**Authors' response**

*The eight miRNAs that were tested by qRT-PCR shown in* Figure 2*were chosen on the basis that they were differentially expressed in our microarray analysis and had previously been shown to be dysregulated in MM or other hematological malignancies. As all of these miRNAs were also significantly differentially expressed when measured by qRT-PCR they were described as being 'consistent with the microarray data'. The only other miRNAs that we measured (miR-32 and miR-181a) by qRT-PCR which were also chosen on the basis shown to be dysregulated in previous studies but we did not find them to be significantly different by qRT-PCR which is why the data was not included in* Figure 2.

4. In the discussion the authors state that the investigators of reference 17 "only examined 10 MM and 4 controls". In the abstract of this manuscript, Pichiorri et al. indicate that 16 samples from patients with MM

were studied along with 6 with MGUS and 6 normal donors. Also, in this same spot of the discussion, the authors indicate that the investigators of reference 16 only studied 2 controls but the 52 patient samples analyzed was not mentioned. Moreover, the purpose of that study was to risk stratification.

*Authors' response*

*These comments in the discussion were made in the context of the identification of aberrantly expressed miRNA associated with MM diagnosis. The Pichiorri study states in the results section (pg 12886) that their MM-associated miRNA signature was derived from the array data of 41 MM cell lines, 10 MM patients and 4 controls. Whereas for the Zhou et al study (pg 7904) the MM miRNA signature was derived from a comparison of array data from 52 MM cases with samples from 2 healthy donors. Whilst it is true that the Zhou et al study mainly focused on the identification of risk groups within the 52 MM cases, a significant part of the results section of this paper refers to the identity of miRNAs that are differentially expressed between controls and MM cases. The point that we were making in the discussion is that insufficient numbers of controls were used in both of these previous studies, and that our study was therefore an improvement on these studies, in the context of the identification of aberrantly expressed miRNAs in MM tumor cells.*

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## Additional material

**Additional file 1: Additional tables (Tables S1-S7) and figure (Figures S1-S4). Table S1**. Clinical details of MM and MGUS patients. **Table S2**. Table of microRNAs aberrantly expressed in DLBCL, SzS and MM, highlighting those that are common. **Table S3**. MicroRNAs differentially expressed ($P < 0.05$) between MGUS (n = 5) and controls (n = 9). **Table S4**. MicroRNAs differentially expressed ($P < 0.05$) between MM (n = 32) and MGUS (n = 5). **Table S5**. MicroRNAs differentially expressed ($P < 0.05$) between IgA (n = 8) and IgG (n = 13) isotype MM cases. **Table S6**. MicroRNAs differentially expressed ($P < 0.05$) between LC-only myeloma (n = 8) and non-LC-only myeloma MM cases (n = 21). **Table S7**. MicroRNAs associated with event-free survival (EFS) in MM cases. **Figure S1**. Heat map depicting cluster analysis of MM and control samples on the basis of expression values of 129 MM-associated microRNAs. **Figure S2**. Heat map depicting cluster analysis of MGUS and control samples on the basis of expression values of 39 MGUS-associated microRNAs. **Figure S3**. Venn-diagram depicting relationship between microRNAs differentially expressed in MGUS, MM and controls. **Figure S4**. Heat map depicting cluster analysis of IgG and IgA isotype MM cases on the basis of expression values of 21 isotype-associated microRNAs (Table S5).

## List of abbreviations

ANOVA: analysis of variance; *BCL2* - B-cell CLL/lymphoma 2; *CCND1* - cyclin D1; *DGCR8* - DiGeorge syndrome critical region gene 8; EFS: event-free survival; *FGFR3* - fibroblast growth factor receptor 3; FISH: fluorescent in situ hybridization; *HGMA1* - Homogentisate 1,2-dioxygenase; IgH: immunoglobulin (heavy chain); *LMO2* - LIM domain only 2 (rhombotin-like 1); *MCL1* - myeloid cell leukemia sequence 1 (BCL2-related); MGUS: monoclonal gammopathy of undetermined significance; MIAME: Minimum Information About a Microarray Experiment; MM: multiple myeloma; PC: plasma cell; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; *RB1* - retinoblastoma 1; SVM: support vector machine; SzS: Sezary syndrome;

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## Authors' contributions

Conceived and designed the experiments: JC CHL CSRH TL JB JSW FP. Performed the experiments: JC EB X-HC DT CHL. Analyzed the data: ST X-HC CHL. Contributed reagents/materials/analysis tools: RK DH NJS. Wrote the paper: CHL JC. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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