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ORIGINAL ARTICLE – CLINICAL ONCOLOGY

Clinical significance of circulatory microRNA-203 in serum as novel potential diagnostic marker for multiple myeloma

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Abstract

Purpose Multiple myeloma (MM) is a hematological malignancy marked by uncontrolled proliferation and accumulation of plasma cells in bone marrow. Despite presence of numerous diagnostic markers for MM, their invasive and non-specific nature demands identification of some effective biomarker. Small non-coding RNAs, i.e., microRNAs being secreted out in circulation could depict the change in homeostasis. Earlier, we reported diagnostic potential of a proteoglycan, Versican (VCAN) in MM, hence, VCAN linked cell-free microRNAs have been explored to study their diagnostic involvement in MM.

Methods Biopsy proven MM patients and controls were recruited. The relative microRNA expression of VCAN linked microRNAs (miR-143, miR-144, miR-199, and miR-203) along with levels of VCAN have been investigated in bone marrow supernatant fluid (BMSF) and blood serum and their correlation were done with clinico-pathological parameters. The diagnostic potential was assessed using ROC curve.

Results Relative microRNA expression of all microRNAs was found significantly lower in MM patients in both BMSF and serum while VCAN levels were substantially higher in patients. VCAN levels showed positive trend while microRNAs expression showed negative trend with severity of disease. miR-203 showed significant correlation with myeloma-associated parameters and also showed optimum sensitivity and specificity for diagnosis of MM in serum.

Conclusions Downregulation of cell-free microRNAs illustrates their importance in MM. The negative trend of microRNAs with disease progression suggests their diagnostic significance. Correlation of miR-203 with myeloma clinical parameters along with optimum sensitivity and specificity affirms its non-invasive diagnostic potential in MM which could further be validated in larger patient cohort.

Keywords Multiple myeloma · miR-143 · miR-144 · miR-199 · miR-203 · Versican · Diagnostic marker

Introduction

Multiple myeloma (MM) is the second most common hematological malignancy after non-Hodgkin lymphoma and is marked by the proliferation followed by the accumulation of malignant plasma cells in the bone marrow (Palumbo and Anderson 2011). MM accounts for 1% of all neoplasms and 13% of all hematological malignancies. This malignancy was first documented in 1844 and since then, research is being constantly carried out to identify some effective and sensitive diagnostic marker for the detection of MM (Kyle and Rajkumar 2008). Undoubtedly, numerous diagnostic methods for MM are available in the present scenario but their non-specific or invasive nature demands the identification of some effective, non-invasive, sensitive and specific biomarker for MM.

microRNAs are small non-coding RNAs involved in the regulation of gene expression in physiological or pathological processes (Bartel 2004). Cell-free microRNAs or circulating microRNAs could behave as the signature of a particular condition and hence, have been emerged as the potential biomarker for various diseases including cancer. But a question arises as of how to decide the basis of selection of few

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microRNAs from a total pool of miRBase for MM. Previously, our research group reported the diagnostic potential of a chondroitin sulfate proteoglycan, Versican (VCAN) in MM and we observed its optimum sensitivity and specificity in the malignancy (Gupta et al. 2015). This proteoglycan is mainly produced in the bone marrow microenvironment but its secretion in blood proposed the utilization of blood as an alternative of bone marrow in MM.

There are certain reports which suggest the regulation of VCAN by small non-coding RNAs, i.e., microRNAs in cancer. Fang et al. reported alteration in levels of endogenous microRNAs in hepatocellular carcinoma after transfecting VCAN 3′UTR which behave as competitive endogenous RNA for microRNAs (Fang et al. 2013). Similar results have also been reported in breast cancer by Lee et al. in which they showed the modulation of microRNAs activities by VCAN 3′UTR fragment (Lee et al. 2010). The regulation of VCAN by miR-203 has also been tested in melanoma cell lines wherein the authors have shown the anti-cancer potential of miR-203 via targeting VCAN (Bu and Yang 2014). Thus, on the basis of above mentioned literatures, miR-143, miR-144, miR-199 and miR-203 have been selected to explore their diagnostic potential in MM.

There are limited studies available showing the significance of these microRNAs in MM. The downregulated expression of miR-144 has been reported in plasma samples of MM patients in a single study and hence, anti-myeloma potential of miR-144 in vitro (Zhao et al. 2017a, b). miR-203 has also shown to be differentially expressed in plasma cells from MM patients and also observed hyper-methylated in myeloma contributing to its downregulation in the disease (Chi et al. 2011; Wong et al. 2011). As per the published literature, no reports are available for miR-143 and miR-199 in clinical samples in MM, but there is a study in MM showing targeting of angiogenesis by miR-199 mimics in myeloma in vitro and in vivo (Raimondi et al. 2014). However, these microRNAs have not been studied yet in the circulatory milieu of MM and, therefore, their clinical importance in terms of diagnosis has not been taken into account till date in MM.

Hence, this study hypothesizes to exploit the diagnostic importance of cell-free microRNAs in MM. To achieve this aim, microRNA expression along with VCAN has been investigated in bone marrow and blood circulation in MM. The expression of microRNAs was correlated with VCAN and also with clinico-pathological parameters of the patients. Lastly, the diagnostic potential of microRNAs was assessed by determining their sensitivity and specificity in MM.

Materials and methods

Study samples

This study has been approved by the Institute's ethics committee and informed written consent was obtained from all the study subjects. 30 newly diagnosed MM patients registered at Department of Hematology, AIIMS, New Delhi were included. The patients were categorized into three stages on the basis of International Staging System (ISS) which includes β2-microglobulin and albumin. In addition, two categories of controls were enrolled which includes 15 patients undergone for bone grafting procedure or spine surgery registered at Department of Orthopedics, AIIMS for bone marrow controls and 30 healthy volunteers for blood controls for precise comparison with patients in both the circulatory milieu. BM aspirate and peripheral blood were obtained from MM patients. Additionally, BM aspirate was taken from the bone marrow controls while peripheral blood sample from blood controls. 2 ml BM aspirate from the study subjects was collected in EDTA vials at the time of their diagnostic procedure and 2 ml blood was withdrawn in plain sterile tubes free of endotoxins. Bone marrow and blood were kept at room temperature for 10 min followed by centrifugation at 600*g* for 10 min for bone marrow supernatant fluid (BMSF) and serum isolation, respectively. Both of the circulatory fluids were stored at − 80 °C for further use.

Enzyme-linked immunosorbent assay (ELISA)

High-sensitive commercially available ELISA kit was used to estimate the circulatory levels of Versican (VCAN) in BMSF/serum samples of the study subjects. VCAN ELISA kit was supplied by USCN Cloud Clone Corporation (Houston, USA). This kit was based on the principle of sandwich ELISA in which a monoclonal antibody against the VCAN antigen had been pre-coated onto the wells of microtiter plates provided in the kit. The standard or samples containing VCAN antigen were incubated with the antibody-coated plates to allow binding of VCAN to the antibody. Following incubation, a primary monoclonal anti-VCAN antibody conjugated to biotin was added. Subsequently, washing was done to remove the unbound antibody and antigen followed by the addition of an avidin-HRP conjugated antibody specific for the primary antibody. After incubation and following a wash, TMB one-step substrate reagent reactive with HRP was added to develop a colored product which was then terminated by adding stop solution containing acid and absorbance was measured at 450 nm. A reference curve was obtained by

plotting different concentrations of standards vs absorbance. The levels of VCAN in unknown samples were calculated from the standard plot.

Quantitative microRNA expression by real-time PCR

Total RNA was extracted from serum and BMSF as per the manufacturer's instructions of miRNeasy serum/plasma isolation kit supplied by Qiagen (Hilden, Germany). Briefly, 200 µl BMSF/serum sample was lysed using 1 ml Qiazol lysis reagent followed by vortexing and incubation at room temperature for 5 min. Then, chloroform was added, vortexed, incubated for 2–3 min and then centrifuged at 15,000*g* for 15 min at 4 °C. After centrifugation, the upper aqueous phase containing RNA was taken followed by the addition of absolute ethanol to precipitate total RNA. The tube was mixed gently to make homogenous suspension which was then transferred into an RNeasy MinElute spin column and centrifuged at 8000*g* for 30 s. The column was then washed with different buffers provided in the kit and RNA was eluted by adding RNase free water into the column, incubated for 15–20 min and centrifuged at 8000*g* for 2 min. The RNA obtained was then quantified using Nano Drop Spectrophotometer and its purity was determined by 260/280 and 260/230 ratio. RNA was stored at − 80 °C until further use.

RNA isolated from the kit was used to synthesize cDNA using miscript II RT kit, Qiagen (Hilden, Germany). Using this kit, microRNAs were specifically polyadenylated and reverse transcribed into cDNA using MMLV reverse transcriptase. cDNA was then used as template in Quantitative PCR (Q-PCR) to determine the relative microRNA expression of miR-143, miR-144, miR-199, and miR-203. The primers specific to the microRNAs were commercially synthesized by Qiagen (Hilden, Germany). *C. elegans* miR-39 was used as an endogenous control (spike in control) for relative quantitation. This microRNA was added into the sample while RNA isolation for normalization. The data were measured by $2^{-\delta Ct}$ method, where Ct values of the molecules were normalized to that of miR-39 and compared with their respective controls.

Correlation analysis

Spearman correlation analysis was performed to determine the correlation between microRNAs and VCAN in both bone marrow and blood circulation. The levels of different microRNAs were also correlated with each other in both the circulatory fluids. Further, levels of microRNAs and VCAN were correlated with various clinico-pathological parameters of myeloma patients which includes hemoglobin, β2-microglobulin, plasma cells, M-band, total protein, albumin, globulin, creatinine, and calcium.

Statistical analysis

Stata 12.0 was used for statistical assessment. Data were presented as median (range). Wilcoxon rank-sum test was applied to statistically analyze the differences between MM patients and controls. Wilcoxon signed rank test was used to identify differences in the levels of microRNAs and versican between BMSF and blood of the same patients. Kruskal–Wallis test was applied for stage-wise analysis of molecules. Spearman's correlation analysis was performed to identify the correlation between VCAN and microRNAs and all the molecules with various clinico-pathological parameters of patients. The diagnostic potential of microRNAs in BM and blood were assessed using receiver operating characteristic (ROC) curve which determines the area under the curve, sensitivity, specificity, and the respective cut-off points. A p value of <0.05 with 95% confidence interval was considered statistically significant.

Results

Study subjects

The total of 30 newly diagnosed biopsy proven MM patients were recruited in the study along with 15 bone marrow controls and 30 healthy blood controls. Their demographic details are shown in Table 1. According to ISS, 4 patients were in Stage I, 14 patients were in Stage II and 12 patients were in Stage III.

Circulatory levels of VCAN in study subjects

The circulatory levels of VCAN were observed significantly higher ($p<0.001$) in MM patients in comparison to controls in both BMSF and blood with considerably elevated expression ($p<0.05$) in BM as compared to blood as shown in Fig. 1 and Table 2. Upon inter-stage analysis, the expression of VCAN showed a trend with disease severity in both the compartments with a gradual increase in levels as the disease progresses. The stage III patients showed significantly enhanced circulatory expression of VCAN than stage I ($p<0.01$) and stage II ($p<0.001$) patients in BMSF. The similar trend has also been observed in blood. Moreover, stage II and stage III patients showed remarkably higher expression ($p<0.001$) of VCAN than controls in blood while stage III patients showed considerable higher expression ($p<0.001$) than controls in bone marrow.

Relative microRNA expression of VCAN linked microRNAs

MicroRNAs linked to VCAN (miR-143, miR-144, miR-199a, miR-203) have been studied by Q-PCR. The relative miRNA expression of all the microRNAs was found

Table 1 Demographic data of multiple myeloma patients and control subjects

Patients		
Total number (*n*)	30	
Blood/bone marrow	30/30	
Male/female	17/13	
Age (years)	59	
Range	(35–76)	
Stage I	4	
Stage II	14	
Stage III	12	
Hb (g/dl)	9.4 ± 2.1	
≤ 10 g/dl	20 (66.7%)	
> 10 g/dl	10 (33.3%)	
β2 microglobulin		
≤ 3.5 mg/l	4 (13.3%)	
> 3.5 mg/l	26 (86.7%)	
Plasma cells (%)	19.2 ± 16.2	
M- band (g/dl)	2.6 ± 2.0	
Total protein (g/dl)	7.7 ± 1.1	
Albumin (g/dl)	3.7 ± 0.9	
Globulin (g/dl)	3.9 ± 1.2	
Creatinine (mg/dl)	0.9 ± 0.5	
Calcium (mg/dl)	8.6 ± 0.6	
	Bone marrow	Blood
Controls		
Total number (*n*)	15	30
Male/female	7/8	22/8
Age (years)	44	44
Range	(25–68)	(33–55)

Values are represented as mean ± SD

significantly lower in MM patients in comparison to controls in both blood ($p < 0.001$) and bone marrow ($p < 0.001$) but the expression was much lower ($p < 0.001$) in blood than bone marrow (Fig. 2; Table 2). This finding could signify the negative relation between VCAN and microRNAs. These miRNAs also showed a negative trend with an increase in disease severity, i.e., relative microRNA expression decreased upon progression of the disease to a higher stage. Though the microRNA levels reduce with increase in stage but only the difference in the expression of miR-203 was significant between stage I and stage III patients and stage II and stage III patients in blood. Furthermore, it has been found that relative microRNA expression of all miRNAs in stage II and stage III patients were significantly lower as compared to controls in both bone marrow and blood as described in Fig. 2 and Table 2.

Correlation analysis

The expression of microRNAs was correlated with VCAN levels in both blood and bone marrow using Spearman's correlation analysis as discussed in Table 3. It has been observed that expression of miR-144, miR-199, and miR-203 showed significant negative correlation with levels of VCAN in bone marrow while miR-144 and miR-203 also showed a significant negative correlation with VCAN in blood. The levels of VCAN and relative miRNA expression of miR-143, miR-144, miR-199, and miR-203 were correlated with various clinico-pathological parameters of MM patients and significant correlation was observed with several such parameters as shown in Table 4. It has been found that expression of miR-203 in blood consistently showed significant association with β2 microglobulin, M-band, and

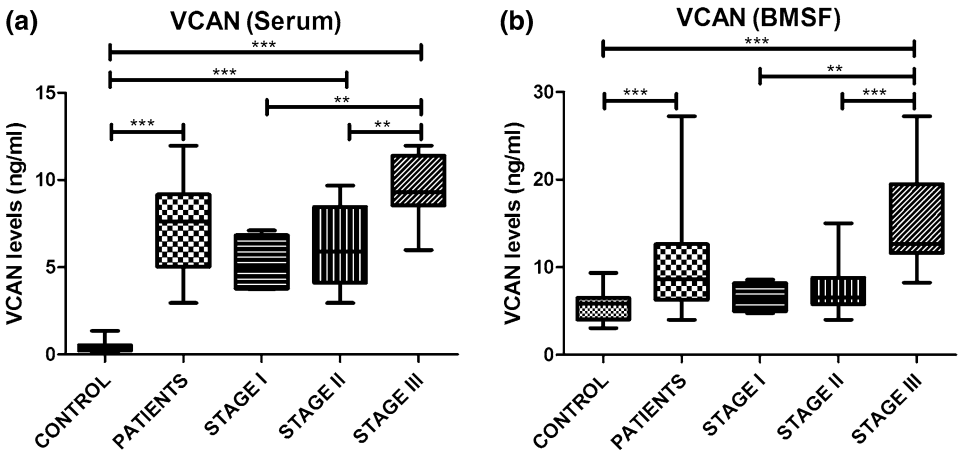


Fig. 1 Circulatory levels of versican in **a** serum and **b** bone marrow supernatant fluid (BMSF) of multiple myeloma patients and controls. The levels were represented as ng/ml. The patients were divided into three stages on the basis of International Staging System. $N = 30$ blood controls; $N = 15$ bone marrow controls; $N = 30$ patients; $N = 4$ stage I patients; $N = 14$ stage II patients; $N = 12$ stage III patients. The bars of significance not mentioned between some groups depict non-significance. [VCAN: Versican; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$]

Table 2 The level of significance of microRNAs and VCAN between different groups in BMSF and serum samples

p	mir-143		mir-144		mir-199		mir-203		VCAN	
	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum
P vs C	< 0.001	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Stage I vs Stage II	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Stage II vs Stage III	ns	ns	ns	ns	ns	ns	ns	< 0.05	< 0.001	< 0.01
Stage I vs Stage III	ns	ns	ns	ns	ns	ns	ns	< 0.01	< 0.01	< 0.01
Stage I vs C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Stage II vs C	< 0.001	< 0.001	ns	< 0.05	< 0.001	< 0.001	< 0.01	< 0.001	ns	< 0.001
Stage III vs C	< 0.01	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
P-Serum vs P-BMSF	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	

VCAN versican, *BMSF* bone marrow supernatant fluid, *P* patients, *C* controls, *p* significance value, *ns* non-significance

globulin which are the important hallmarks for MM. In addition, the expression of miR-203 in bone marrow correlated significantly with globulin and plasma cells. Moreover, levels of miR-203 in blood showed a significant positive correlation with levels in bone marrow suggesting that blood could be exploited as a true representation of bone marrow and hence, non-invasive detection of miR-203 could be of significance for better diagnosis of MM.

Diagnostic potential of microRNAs in MM

The diagnostic potential of microRNAs was assessed by plotting ROC curve which provides sensitivity, specificity, area under the curve (AUC) and cut-off values of these molecules as shown in Fig. 3. As reported earlier, VCAN showed optimum sensitivity and specificity. In addition to VCAN, expression of miR-203 in blood showed the best sensitivity, specificity and area under the curve out of all microRNAs.

Discussion

With the advent of science, numerous diagnostic methods are available for the detection of MM, but the non-specific nature of β2 microglobulin, specific but invasive property of plasma cell determination in bone marrow prompts researchers to identify some sensitive, specific and non-invasive biomarker for MM. Previously, we reported the diagnostic importance of a chondroitin sulfate proteoglycan, VCAN in MM (Gupta et al. 2015) and this finding formed the basis of selection of cell-free microRNAs to explore their clinical utility for MM diagnosis. There are few reports available describing certain microRNAs linked to the regulation of VCAN in vitro in solid tumors (Fang et al. 2013; Lee et al. 2010; Bu and Yang 2014). But if this regulating property of microRNAs is helpful in the diagnosis of MM as a non-invasive mean has been addressed in the present work. To address this question, VCAN linked microRNAs (miR-143, miR-144, miR-199, and miR-203) were studied along with VCAN in the bone marrow and blood samples of study subjects. These microRNAs have been studied in clinical samples of other cancers, but not a single report is available in circulatory milieu of MM patients. This is the first study assessing the expression of the above-listed microRNAs in MM.

The circulatory levels of VCAN in bone marrow supernatant fluid (BMSF) and blood serum were significantly higher in MM patients in comparison to controls with considerably higher levels in BMSF than serum. This finding is in concordance with our previous report published in 2015 (Gupta et al. 2015).

The relative cell-free microRNA expression levels of all the microRNAs (miR-143, miR-144, miR-199, and miR-203) were determined in BMSF and serum samples and observed that cell-free expression of all microRNAs was significantly lower in MM patients as compared to controls. The decreased expression of miR-143 has also been reported in solid tumors such as bladder cancer and squamous cell carcinoma as well as in hematological malignancies such as leukemia, but no report is available in MM (Motawi et al. 2016; Gao et al. 2011; Elhamamsy et al. 2017; Zhang et al. 2011). There are some controversial reports available showing increased expression of miR-143 in patients with gastric cancer and lymphoblastic leukemia which could suggest the disease-specific role of miR-143 (Obermannova et al. 2018; Piatopoulou et al. 2018).

Similarly, in concordance with our results, miR-144 has been reported to have downregulated expression in cancer patients (Lario et al. 2018; Liu et al. 2017; Liang et al. 2017; Zhao et al. 2017a, b). There is an opposing report available illustrating the enhanced expression of miR-144 in acute myeloid leukemia patients (Sun et al. 2017). There is only a single study published in MM showing the decreased expression in plasma samples of MM patients, and hence, anti-myeloma potential of miR-144 in vitro, but the expression of miR-144 in bone marrow has not been taken into

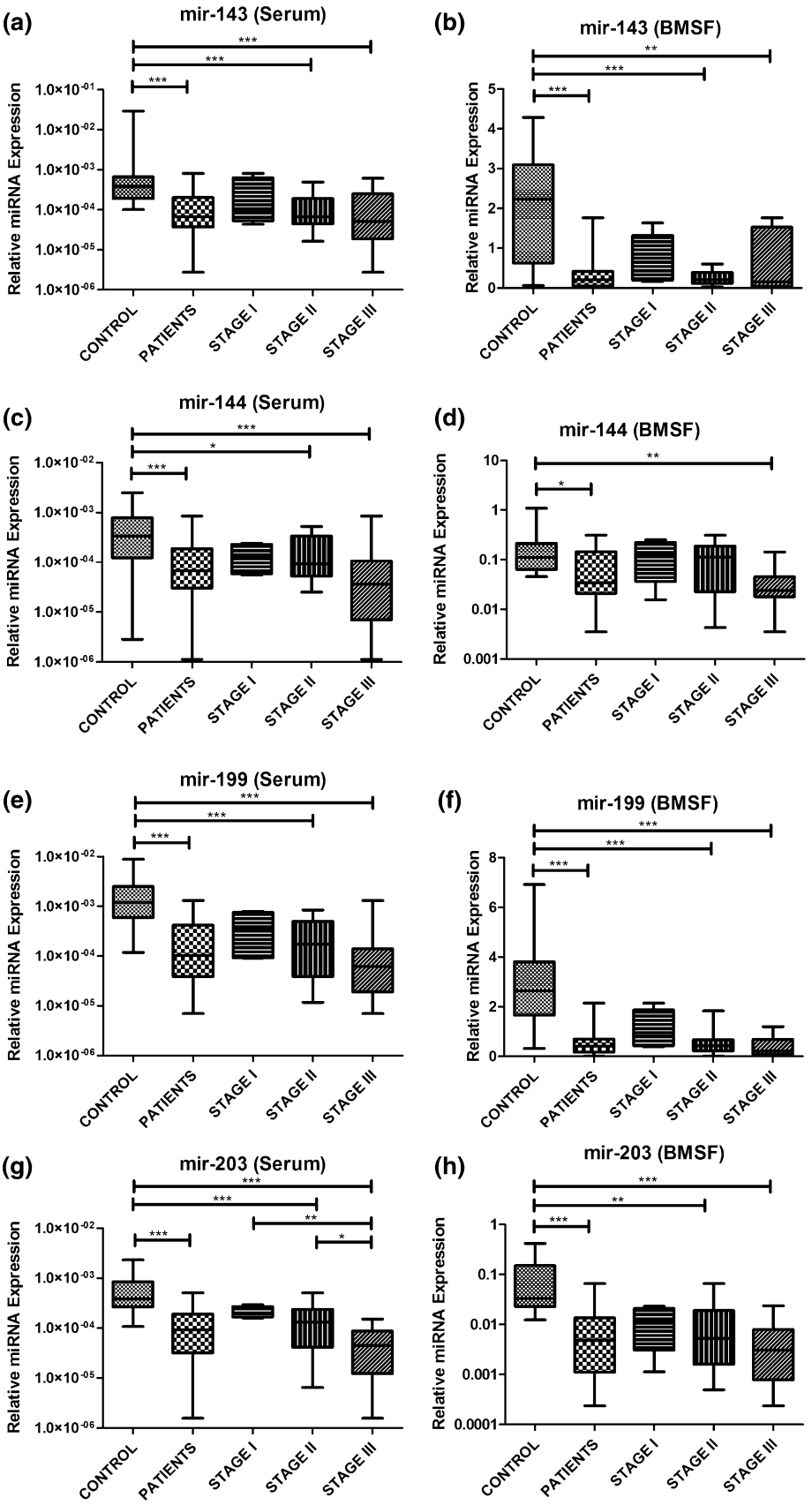


Fig. 2 Box-whisker plot showing relative microRNA expression of **a, b** miR-143, **c, d** miR-144, **e, f** miR-199 and **g, h** miR-203 in serum and bone marrow supernatant fluid (BMSF), respectively, of multiple myeloma patients and controls. The patients were categorized into three stages on the basis of International Staging System. $N = 30$ blood controls; $N = 15$ bone marrow controls; $N = 30$ patients; $N = 4$ stage I patients; $N = 14$ stage II patients; $N = 12$ stage III patients. The bars of significance not mentioned between some groups depict non-significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Table 3 Spearman correlation analysis of microRNAs and VCAN with each other in BMSF and serum samples of MM patients

r (*p*)	mir-143		mir-144		mir-199		mir-203		VCAN	
	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum
mir-143										
BMSF	–	0.056 (0.770)	**0.433 (0.017)**	–	0.249 (0.184)	–	0.317 (0.088)	–	−0.274 (0.143)	–
Serum	–	–	–	0.311 (0.095)	–	**0.709 (<0.001)**	–	**0.605 (<0.001)**	–	−0.293 (0.116)
mir-144										
BMSF			–	0.137 (0.470)	0.086 (0.652)	–	0.228 (0.225)	–	**−0.426 (0.019)**	–
Serum			–	–	–	**0.396 (0.030)**	–	**0.638 (<0.001)**	–	**−0.398 (0.029)**
mir-199										
BMSF					–	0.276 (0.140)	**0.401 (0.028)**	–	**−0.427 (0.019)**	–
Serum					–	–	–	**0.697 (<0.001)**	–	−0.327 (0.078)
mir-203										
BMSF							–	**0.419 (0.021)**	**−0.479 (0.007)**	–
Serum							–	–	–	**−0.638 (<0.001)**
VCAN										
BMSF									–	**0.693 (<0.001)**
Serum										–

The table also includes correlation between BMSF and serum levels of microRNAs and VCAN

Values in parentheses showed *p* values. Bold values represent significant correlation

MM multiple myeloma, *VCAN* versican, *BMSF* bone marrow supernatant fluid

Table 4 Spearman correlation analysis of microRNAs and VCAN levels in BMSF and serum samples with clinico-pathological parameters of myeloma patients

r (*p*)	mir-143		mir-144		mir-199		mir-203		VCAN	
	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum	BMSF	Serum
Hb	0.242 (0.198)	0.181 (0.340)	0.164 (0.387)	0.192 (0.309)	**0.375 (0.041)**	0.091 (0.629)	0.288 (0.122)	0.099 (0.602)	−0.351 (0.057)	−0.270 (0.150)
β2-MG	−0.147 (0.438)	−0.186 (0.326)	−0.180 (0.343)	−0.304 (0.102)	−0.277 (0.138)	−0.315 (0.090)	−0.256 (0.173)	**−0.482 (0.007)**	**0.607 (<0.001)**	**0.470 (0.009)**
Plasma cells	−0.176 (0.352)	0.185 (0.328)	−0.294 (0.115)	**−0.399 (0.029)**	−0.355 (0.055)	−0.020 (0.914)	**−0.585 (<0.001)**	−0.280 (0.135)	**0.679 (<0.001)**	**0.569 (<0.001)**
M-band	−0.009 (0.961)	−0.130 (0.492)	−0.062 (0.744)	**−0.397 (0.030)**	−0.280 (0.134)	−0.336 (0.070)	**−0.549 (0.002)**	**−0.369 (0.045)**	0.178 (0.348)	0.339 (0.068)
T. Protein	0.227 (0.229)	−0.285 (0.127)	0.304 (0.102)	−0.110 (0.561)	0.116 (0.541)	−0.280 (0.134)	−0.160 (0.397)	−0.074 (0.698)	−0.068 (0.720)	0.029 (0.679)
Albumin	0.277 (0.138)	−0.203 (0.283)	**0.455 (0.011)**	0.060 (0.755)	0.252 (0.180)	−0.014 (0.943)	**0.403 (0.027)**	0.309 (0.097)	−0.352 (0.056)	−0.201 (0.286)
Globulin	−0.003 (0.988)	−0.152 (0.423)	−0.105 (0.581)	−0.270 (0.150)	−0.167 (0.379)	−0.251 (0.180)	**−0.467 (0.009)**	**−0.438 (0.016)**	0.270 (0.149)	0.274 (0.142)
Creatinine	−0.264 (0.159)	**−0.412 (0.024)**	−0.183 (0.333)	−0.266 (0.156)	0.259 (0.167)	0.098 (0.607)	0.071 (0.708)	−0.331 (0.073)	0.138 (0.467)	0.133 (0.482)
Calcium	0.314 (0.090)	−0.020 (0.918)	0.005 (0.980)	0.085 (0.657)	−0.087 (0.648)	0.152 (0.421)	0.202 (0.284)	−0.104 (0.584)	0.250 (0.183)	0.079 (0.680)

Values in parentheses showed *p* values. Bold values represent significant correlation

VCAN versican, *BMSF* bone marrow supernatant fluid, *β2-MG* β2-microglobulin, *Hb* hemoglobin

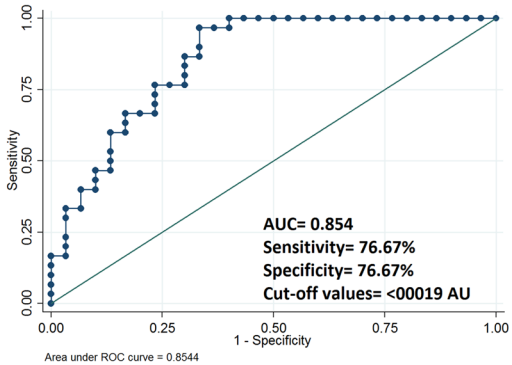
Fig. 3 Receiver operating characteristic (ROC) curves of **a, b** miR-143, **c, d** miR-144, **e, f** miR-199 and **g, h** miR-203 in serum and bone marrow supernatant fluid (BMSF), respectively. This curve determines AUC, sensitivity, specificity, and cut-off values mentioned in the respective graphs (*AUC* area under the curve, *AU* arbitrary unit)

account so far (Zhao et al. 2017a, b). Similar to our findings, lower expression of miR-199 has been described in bladder cancer and leukemia (Sakaguchi et al. 2018; Troppan et al. 2015; Luna-Aguirre et al. 2015), but no report is discussed in myeloma patients. There is a single study depicted in MM in which miR-199 mimics targeted angiogenesis in myeloma in vitro and in vivo (Raimondi et al. 2014). Besides, miR-203 expression was observed to be downregulated in both solid tumors and blood cancers (Zheng et al. 2017; Chen et al. 2017; Dusílková et al. 2017). There are few studies available showing differential expression of miR-203 in myeloma cells and also reported hyper-methylation of miR-203 gene at the promoter region in plasma cells of myeloma patients (Chi et al. 2011; Wong et al. 2011). Further, an in vitro study suggesting anti-myeloma potential of miR-203 has been published by Wu et al. (2016). However, the expression of miR-203 in circulation of bone marrow and blood has not been documented in MM till date.

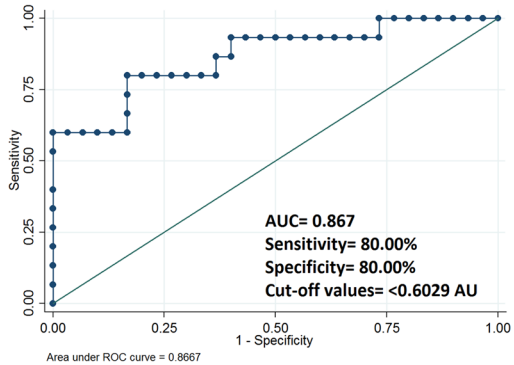
Upon inter-stage analysis, we have observed the positive trend of VCAN while the negative trend of all circulating microRNAs with the severity of disease. These findings could suggest the diagnostic ability of VCAN and cell-free microRNAs in MM. Using Spearman correlation analysis, we have observed a significant negative correlation between VCAN and miR-144, miR-199 & miR-203 in bone marrow circulation while miR-144 and miR-203 expression also showed a significant negative correlation in blood. Moreover, expression of VCAN and microRNAs in both BMSF and serum were correlated with clinico-pathological parameters of patients. It has been observed that levels of cell-free miR-203 showed significant negative correlation with β2 microglobulin which is involved in the staging of myeloma patients. Moreover, serum levels of miR-203 also showed a significant negative correlation with M-band and globulin which are the critical hallmarks for MM. In addition, expression of miR-203 in BMSF correlated significantly with globulin and plasma cells which formed the basis of MM. Besides, serum levels of miR-203 positively correlated with expression in bone marrow circulation affirming the utilization of blood as an accurate representation of bone marrow and hence, non-invasive detection of circulatory miR-203 in serum could be of clinical significance for better and effective diagnosis of MM.

Moreover, the diagnostic significance of microRNAs was determined by plotting ROC curve. It has been found that miR-203 showed better sensitivity, specificity and area under the curve in non-invasive blood specimen of MM patients.

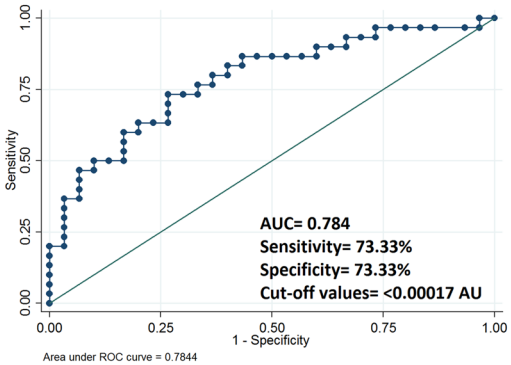
(a) mir-143 (Serum)



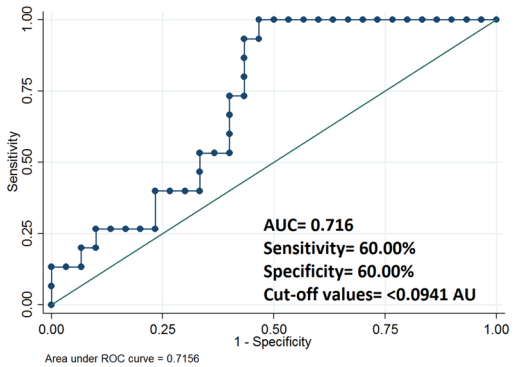
(b) mir-143 (BMSF)



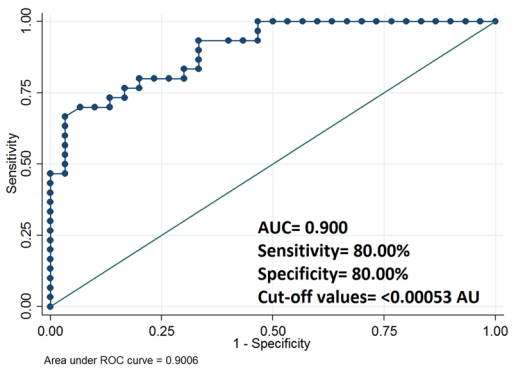
(c) mir-144 (Serum)



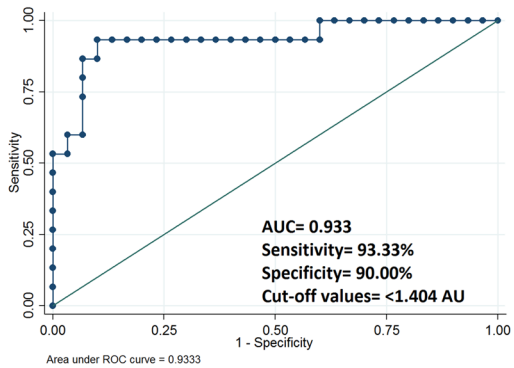
(d) mir-144 (BMSF)



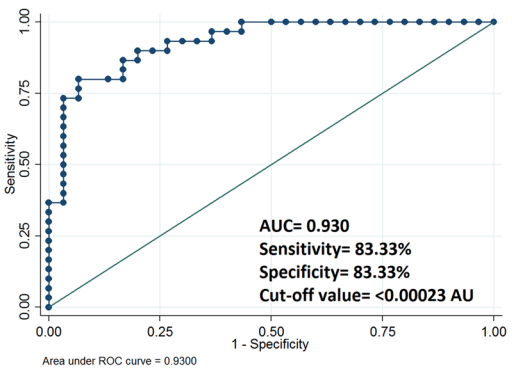
(e) mir-199 (Serum)



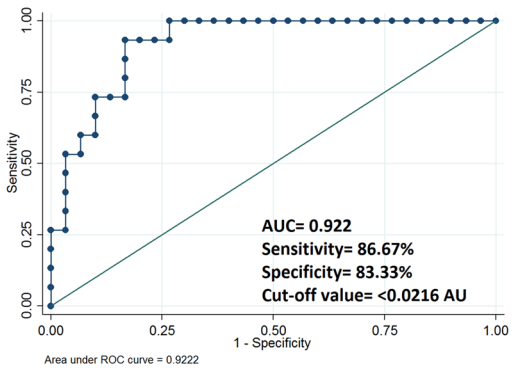
(f) mir-199 (BMSF)



(g) mir-203 (Serum)



(h) mir-203 (BMSF)



In concordance with our findings, the diagnostic ability of miR-203 has also been reported in T-cell lymphoma, but no such data is available in MM (Dusílková et al. 2017).

In conclusion, microRNAs are small non-coding RNAs which are secreted in circulation and could depict the scenario of physiology or pathology condition. Several cell-free microRNAs (miR-143, miR-144, miR-199, and miR-203) related to VCAN have been studied in this maiden attempt and found downregulated in MM patients in both bone marrow and blood circulation. These microRNAs also showed a negative trend with progression of the disease, hence, could be employed as a diagnostic marker for MM. Further, expression of microRNAs was negatively correlating with VCAN, and upon correlation with clinico-pathological parameters of myeloma patients, miR-203 consistently showed significant association with myeloma-associated parameters affirming its potential involvement in the malignancy. Besides, ROC curve analysis showed optimum sensitivity and specificity of miR-203 for diagnosis of MM in serum. This finding could suggest the exploitation of miR-203 as a non-invasive diagnostic biomarker for the better and effective detection of MM after validation in a larger patient cohort to be established in clinical settings in the future.

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Data availability All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee or comparable ethical standards.

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