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# The Role of Local Bone Marrow Renin-Angiotensin System in Multiple Myeloma

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Abstract
Angiotensin II acts as a growth promoter or angiogenic factor via type 1 receptors (AGTR1) in certain tumors. In this study, we examined the bone marrow AGTR1 expression in multiple myeloma (MM) and its relationship with the regulation of angiogenesis and prognostic factors. Bone marrow *AGTR1* mRNA levels of 39 MM patients and 15 healthy controls were analysed with quantitative RT-PCR. Bone marrow angiogenesis was evaluated with immunohistochemical measurement of tissue vascular endothelial growth factor (VEGF), CD34 and factor VIIIrAg (fVIIIrAg). Patients had higher bone marrow VEGF, fVIIIAg, CD34 and AGTR1 expression levels when compared to controls, and patients with severe-diffuse bone marrow infiltration showed higher bone marrow VEGF, fVIIIAg, CD34 and *AGTR1* mRNA levels when compared to other patients. *AGTR1* expression was found positively correlated with plasma beta-2 microglobulin level and patients with increased *AGTR1* expression showed increased bone marrow CD34 levels.

Key words: Multiple myeloma, renin-angiotensin system, angiotensin type 1a receptor

Introduction

Side effects of angiotensin converting enzyme inhibitors (ACEI) such as anemia and leukopenia indicated inhibitory effects of these drugs on normal bone marrow hematopoesis. With the previous data about locally acting growth factor like effects of angiotensin II (ATII), Haznedaroglu et al. first mentioned a local renin-angiotensin system (RAS) in the bone marrow [1,2]. Possible role of the bone marrow RAS has been reported in acute myeloid leukemia later [3,4]. Abali et al. showed increased bone marrow ACE level compared to serum ACE in acute leukemia [5].

ATII was also related with angiogenesis, which could be inhibited with ACEI and ATII type 1a receptor (AGTR1) antagonists [6-8]. It can induce neovascularization due to increased expression of different growth factors (angiopoietin 2, vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet derived growth factor, transforming growth factor beta and epidermal growth factor), nitric oxide synthase and metalloproteinases [9]. Tamarat et al. showed increased neovascularization with subcutaneous ATII injection in a rat model, which was found well correlated with serum VEGF, and endothelial nitric oxide levels. AGTR1 antagonists and VEGF neutralizing antibodies completely prevented the ATII-induced angiogenesis [6]. In another study, ATII was related with tumoral enlargement by inducing angiogenesis and malignant cell proliferation via AGTR1, while ACEI was shown to decrease cancer risk [8]. Egami et al. Compared Agtr1+ and Agtr1- malignant melanoma rats and found decreased tumor angiogenesis and doubling time in Agtr1- rats, which resulted with increased survival rate. AGTR1 antagonist showed supressed tumor growth in Agtr1+ rats [10].

Interaction between malignant plasma cells and bone marrow microenvironment is important in the etiopathogenesis of multiple myeloma (MM). Increased angiogenesis was shown in the bone marrow microenvironment which was related with the disease progression, resistance to treatment and worse prognosis [11]. Tumor growth and angiogenesis may result from various cytokines and factors. VEGF is the most well characterized pro-angiogenic factor produced by myeloma cells. It also stimulates stromal cells to produce IL-6, which is a potent myeloma growth factor [12,13].

The aim of this study is to find out any possible relationship between local bone marrow RAS activity and MM. Bone marrow RAS activity of the patients were compared with their disease activity and bone marrow angiogenesis.

Materials and methods

*Patients and controls*

De novo multiple myeloma patients (n=39) without any previous treatment were enrolled into the study group. Control group (n=15) included healthy bone marrow donors and those with normal bone marrow histology who were examined clinically with any other reason (Fig.1). Patients and controls those taking drugs (ACE inhibitor, AGTR1 antagonist, beta blocker, spiranolactone) with possible effects on RAS were excluded. Patients and controls with acute and/or chronic infectious diseases, inflammatory rheumatoid diseases and any other cancer were excluded. The ethical committee of Istanbul Medical Faculty (reference number 2008/305) approved this study and written informed parental consents were obtained.

*RNA isolation and cDNA synthesis*

Bone marrow samples were collected in 2 mL ethylene di amine tetra acetic acid tubes. By using white blood cells, total RNA was isolated according to the manufacturer's instructions (QIAGEN, Germany). RNA quality and quantity was measured by spectrophotometer (ND-1000, NanoDrop Technologies, Inc., USA), and 1 µg of total RNA was used. Random primers (20 µM, Roche Diagnostics, Germany), 10 mM dNTP set (Fermentas UAB, Lithuania), RiboLock RNase Inhibitor (20 U/µL, Fermentas) and Moloney murine leukemia virus reverse transcriptase (200 U/µL, Fermentas) were used for cDNA synthesis. cDNA samples were stored at -20 °C.

*Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis*

Real-time quantitative PCR was carried out on the Light Cycler 480 Instrument (Roche Applied Sciences, Manheim, Germany). The specific primer probe sets are given at Table 1. Real-time amplification was performed with Light Cycler 480 Probe Master Mix (Roche) as described in the protocol. Real-time amplification was performed with a final reaction mixture of 20 µL containing 5 µM of each primer, 0.5 µM of each probe, LightCycler 480 Probe Master Mix, and 100 ng/µL of cDNA. For normalization, the most stable three genes (*B-ACTIN, CYCLOPHILIN A* and *ABL*) were selected by GeNorm (V3.4, Amel, Belgium) software (Table 1). Each sample was studied in duplicates and all runs were repeated twice. The PCR protocol was as follows: initial denaturation at 95 °C for 7 min, and amplification segment at 5 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C for 45 cycles. Relative expressions were calculated according to the ΔΔCt method, based on the mathematical model described by Livak et al [14].

*Immunohistochemical studies*

Bone marrow angiogenesis was evaluated with the immune histochemical measurement of tissue vascular endothelial growth factor (VEGF) (Fig.2), CD34 (Fig.3) and factor VIIIrAg (fVIIIrAg) indexes.

Bone marrow biopsy samples were fixed in formalin (10%) and then embedded into tissue paraffin blocks. After staining with haematoxylin-eosin, they were examined under microscope. Tissue samples were incubated with anti VEGF mouse monoclonal antibodies (clone G153-694) at 2 µg antibody/ml dilution. Immune cytochemical streptavidin-biotin peroxidase complex was used in the next stage followed by diaminobenzidine (DAB) chromogen for visualization of peroxidase reaction. Immune histochemical stain activity was estimated semiquantitatively by using the immunoreactive score [15]. Scores were given to intensity of the reaction (stain) (0 to 3) and percentage of the cells with positive reaction (0 to 4). Then final score was obtained with the multiplication of the both scores (0 to 12) (Table 2).

Staining of vascular endothelial cells with anti-CD34 murine monoclonal antibodies (Clone QBEnd/10, Neomarkers, CA, USA) at 1/100 dilution was also used to show bone marrow angiogenesis as described by Perez-Atyde et al. [16]. Bone marrow biopsy samples were examined at x80 magnification and five distinct fields were selected for evaluation. Mean number of CD34 stained vascular structures was defined as number of vessels/mm$^2$. FactorVIIIrAg was also used to demonstrate bone marrow angiogenesis. (Table 3)

Serum ACE levels of the both groups were measured with sandwich ELISA method. One micro plate was coated with an ACE specific monoclonal antibody. Standards and serum samples were put in small eppendorfs and ACE was bound with immobilized antibodies. After unbound materials were washed out of the wells, ACE specific enzyme-linked polyclonal antibody was

added to eppendorfs. Later, unbound antibody-enzyme particles were washed out which was followed by addition of substrate solution to samples. Color change occured in eppendorfs that was parallel with the ACE levels.

*Statistical Analysis*

SPSS 15.0 statistical software was used to analyse data. Continuous variables were described by the use of statistical characteristics (means, standard deviations, median). Discrete variables were described as counts and percentages. Independent Samples *t*-test, Chi-square test and Pearson correlation analysis were used during evaluation of the results. Differences between two groups were assessed by the Mann-Whitney U test. A p value of $\leq 0.05$ was considered as statistically significant.

## Results

*AGTR1 mRNA expression in MM cases and controls*

Thirty-nine MM patients (male/female: 20/19) were enrolled into the study. Control group included five healthy bone marrow donor and 10 people with normal cellular bone marrow biopsy who were examined for any other reason (male/female: 8/7). Mean ages of the patients and controls were 63±10 (44-81) and 49±14 (27-80) years respectively. Clinical characteristics of the patients were given in Table 4. Bone marrow VEGF, CD34 and factor VIII rAg and *AGTR1* mRNA expression levels of the patients were given in Table 5. MM patients had higher bone marrow VEGF, CD34 and fVIIIrAg levels and increased *AGTR1* mRNA expression levels when compared to controls (Table 6). Patients with severe-diffuse bone marrow infiltration pattern showed higher bone marrow VEGF, CD34 and factor VIII rAg levels and higher bone marrow *AGTR1* mRNA expression when compared to others with mild-patchy infiltration pattern (Table 7, Fig.4). Plasma B2M concentrations of the patients were found well correlated with their bone marrow *AGTR1* mRNA expression levels (Fig.5, p=0.002). No association was found between disease stage and bone marrow *AGTR1* mRNA expression (p=0.76). Serum ACE levels of MM patients did not show any significant difference when compared to control group.

Patients with higher *AGTR1* mRNA expression showed increased bone marrow CD34 (p=0.011 by Mann Whitney test). Similarly, patients with higher *AGTR1* mRNA expression showed increased bone marrow VEGF and fVIIIrAg indexes although they did not reach statistical significance (p=0.080, and p=0.3, respectively).

## Discussion

Renin-angiotensin system (RAS) has an attracted attention because of its physiological and therapeutic potential. An extensive transcriptomic meta-analysis showed the expression patterns of RAS system members in normal human tissues, including hematopoietic cells and bone marrow stem cells. AGT ligand was determined expressed almost all tissue types indicating its physiological importance. Bone marrow derived cells have prominent expression of classical systemic RAS participants (*AGT- REN-ACE-AGTR1*) and they have almost the same expression pattern that is indicating the transcriptional coordination may be preserved during "cell lineage" [17].

RAS system plays role in hematopoietic stem cell plasticity. There is increasing number of evidence that deregulated local bone marrow RAS could play role in malignant transformation by increasing cellular proliferation and differentiation. ACE, induces bone marrow stem cells to enter S-phase through increasing hydrolysis of acetyl-N-Ser-Asp-Lys-Pro (AcSDKP), which

inhibits the proliferation of bone marrow stem cells [18,19]. Conversely, ACE inhibitors (ACEI) increase plasma AcSDKP and downregulate hematopoiesis [20]. ACEI treatment significantly decreased haematocrit level of a patient with polycytemia vera [21].

Wulf et al. showed renin like activity in leukemic blast cells of a patient, and isolated renin like peptide from myeloblasts [3,4]. Abali et al. compared serum and bone marrow ACE concentrations of newly diagnosed acute leukemic patients and found significant increase in the latter. Serum ACE levels were found correlated with bone marrow infiltration rate and the number of blasts in the peripheral blood [5]. RAS members' expressions were detected in different myeloid blast cells [22, 23]. RAS and NOTCH pathways are in communications, and *RBP-J (Recombination Signal Binding Protein For Immunoglobulin Kappa J Region)* gene is an important transcriptional regulator of NOTCH pathway. *Rbp-j* deleted mouse model showed *Ren* expression leading to leukemogenesis in B-cell progenitors. Moreover, there is limited data showed *RBP-J* gene mutations in leukemia patients [24,25]. Serum ACE was found increased in multiple myeloma patients and local RAS components were also found in the following studies [26,27].

ATII plays a fundamental role in controlling cardiovascular function and renal homeostasis. It has many physiologic effects other than regulating vascular tone, such as hormone secretion, tissue growth and neural activity. It has four receptors. ATII type 1 receptor (AGTR1) stimulation activates intracellular pathways that finally lead to vasoconstriction, inflammation and proliferation [9]. Like other cytokines, ATII was shown to use JAK-STAT pathway (*JA*nus or *J*ust *A*nother *K*inase-*S*ignal *T*ransducers and *A*ctivators of *T*ranscription) in the regulation of hematopoiesis [28]. Gomez et al. revealed ability of rat leukocytes to produce angiotensinogen and ATII [29] and Crabos et al. found Agtr1's on thrombocytes [30]. Rodgers et al. showed Agtr1's on CD34+CD38- and CD34+CD38+ cells, lymphocytes and bone marrow stromal cells, and reported increased bone marrow stem cell proliferation with ATII that was inhibited with an AGTR1 antagonist losartan [31]. Mrug et al. reported similar effects of local bone marrow RAS on the erythroid cell lineage [32]. Jokubaitis et al. identified a 160 kDa cell surface glycoprotein BB9 that is found on hematopoietic stem cells (HSC) throughout hematopoietic development, even at the earliest definitive phases. They demonstrated that BB9 monoclonal Ab identifies the somatic form of angiotensin-converting enzyme (ACE/CD143), which suggested its expression by HSCs from primitive phases to the adulthood. Then ACE/CD143 may play role in the regulation of hematopoietic cells [33].

According to our results, bone marrow *AGTR1* expression of our patients showed positive correlation with their bone marrow infiltration pattern and serum B2M levels. Serum B2M level and the morphology of myeloma cells are reliable prognostic factors in MM. Moreover serum B2M was found as the most important parameter in predicting high risk patients [34,35]. Then positive correlation between bone marrow *AGTR1* mRNA levels, bone marrow morphology and plasma B2M provided that bone marrow *AGTR1* expression can give information about prognosis in MM.

Increased bone marrow VEGF, CD34 and fVIIIrAg indexes in our patients reflected neovascularization. Advancing age is associated with the development of vascular endothelial dysfunction. Vascular oxidative stress increases with age as a consequence of greater production of reactive oxygen species (e.g. superoxide) without a compensatory increase in antioxidant defences. In our study the control group seems to be younger than the MM group but it is within age range (mean age 27-80 years) at which MM develops. Patients with higher *AGTR1* expression showed increased bone marrow CD34 index. To our opinion, statistically non-

significant increase in the bone marrow VEGF and fVIIIrAg indexes of these patients was related with the number of subjects enrolled into the study.

Bone marrow *AGTR1* mRNA expression was 2-4% in 5 patients and >4% in 8 patients. Three patients had extremely high levels of expression (67.29%, 66.67% and 61.02%) when compared to others. First patient was a 69 year-old man with stage 3 IgG, kappa light chain MM. He had 30% bone marrow infiltration and his serum monoclonal band, lactate dehydrogenase (LDH) and B2M were 5.28 g/dl, 412 IU/l and 8.83 ng/ml respectively. Second patient was a 51 year-old woman with stage 3 kappa light chain secreting disease. Her laboratory analysis revaled overt disease activity (76% bone marrow infiltration, serum monoclonal band 6.9 g/dl, LDH 696 IU/l and B2M 75.76 ng/ml). Third patient was a 67 year-old woman with stage 2 IgA, lambda light chain secreting disease. She had mild disease activity at diagnosis (21% bone marrow infiltration, serum monoclonal band 1.94 g/dl, LDH 264 IU/l and B2M 2.24 ng/ml). Bone marrow VEGF, CD34 and fVIIIrAg indexes of these three patients were found significantly increased also.

Only 3 control subjects showed 2-4% bone marrow *AGTR1* mRNA expression. Two of them were under medical examination for another reason and showed normocellular bone marrow histology, and the third one was healthy bone marrow donor. Moreover, they had relatively low levels of bone marrow VEGF, CD34 and fVIIIrAg indexes when compared with myeloma patients.

A clinically relevant aspect of the interactions of MM plasma cells in the bone marrow microenvironment is neovascularization, a constant hallmark of disease progression. Myeloma plasma cells also induce angiogenesis indirectly via recruitment and activation of stromal inflammatory cells (i.e. macrophages and mast cells) to secrete their own angiogenic factors. Activating mutations of RAS signalling pathway have been reported in approximately half of newly diagnosed MM and an even higher proportion in relapsed/refractory MM (RRMM) [36,37] which could correlate with ACE expression level. Both findings may provide ACE inhibitors or mitogen-activated protein kinase (MEK) inhibitors usage in MM.

Limitation of the study was the low number of MM patients enrolled into the study which was caused by the planned time schedule and difficulty to find de novo myeloma patients.

In conclusion, bone marrow *AGTR1* expression can give information about bone marrow morphology and can predict disease progression in MM. Further studies are needed to ascertain such an association.

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Table 1: Primers and probes used in qRT-PCR

| **Gene** | **Forward (5'→3')** | **Reverse (5'→3')** | **UPL prob no** |
|---|---|---|---|
| *AGTR I* | ccattatgagtcccaaaattcaa | aaaggaaacaggaaacccagta | 23 |
| *ABL* | cagagaaggtctatgaactcatgc | ggtggatttcagcaaaggag | 86 |
| *CYPA* | cctaaagcatacgggtcctg | cactttgccaaacaccacat | 48 |
| *B ACTIN* | aggcccctctgaacccta | ggggtgttgaaggtctcaaa | *β ACTIN* |

Table 2. Estimation of the bone marrow VEGF index with immunoreactive score

| Score | Positive cells (%) | Intensity of the reaction |
|---|---|---|
| 0 | negative | negative |
| 1 | <10 | weak |
| 2 | 10-50 | moderate |
| 3 | 51-80 | strong |
| 4 | >81 | very strong |

Table 3: Bone marrow immunohistochemical studies of angiogenesis

| Primary antibody | Producer | Antigen retrieval | Primary antibody dilution | Incubation time | Incubation temperature | Amplification system | Chromogen |
|---|---|---|---|---|---|---|---|
| VEGF | Neomarker | EDTA irradiation in microwave oven | 1/50 | 1 hr | Room temperature | Biotin-streptavidin-peroxidase | AEC |
| CD34 | CellMargue | Boiling in citrate buffer under high pressure | 1/50 | 1 hr | Room temperature | Biotin-streptavidin-peroxidase | AEC |
| Factor VIII rAg | Neomarker | Boiling in citrate buffer under high pressure | 1/50 | 1 hr | Room temperature | Biotin-streptavidin-peroxidase | AEC |

Table 4: Clinical characteristics of the patients

| Age (years) | Mean ± SD |
|---|---|
| Male | 64 ± 10 (44 - 81) |
| Female | 63 ± 9 (45 - 80) |
| Total | 63 ± 10 (44 - 81) |
| **Durie-Salmon classification** | **No. of patients** |
| IA | 2 |
| IIA | 9 |
| IIB | 2 |
| IIIA | 16 |
| IIIB | 10 |
| **Paraprotein type** | **No. of patients** |
| IgG | 24 |
| IgA | 7 |
| IgD | 2 |
| Light chain | 6 |
| **Bone Marrow infiltration pattern** | **No. of patients** |
| Patchy | 15 |
| Diffuse | 24 |

Table 5. Patient group; Characteristics, bone marrow proangiogenetic factors and AGTR1 mRNA relative expression.

| No | Sex | Age | PP | Stage | B2M | VEGF | CD34 | fVIIIrAg | AGTR1 mRNA |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 73 | IgG kappa | IIIB | 9,08 | 12 | 21,5 | 50,2 | 1,57 |
| 2 | M | 76 | IgG lambda | IIIB | 9,30 | 12 | 57,6 | 74,0 | 0,87 |
| 3 | M | 75 | IgA lambda | IIIA | 11,00 | 12 | 153,0 | 54,8 | 1,21 |
| 4 | F | 64 | IgG kappa | IIA | 1,45 | 6 | 33,0 | 19,4 | 0,85 |
| 5 | F | 60 | IgG lambda | IIIA | 11,49 | 8 | 50,0 | 36,8 | 2,64 |
| 6 | M | 63 | IgG kappa | IIIA | 3,63 | 12 | 76,0 | 188,2 | 0,79 |
| 7 | F | 80 | IgG kappa | IIA | 8,09 | 3 | 20,0 | 23,4 | 0,86 |
| 8 | M | 69 | IgG kappa | IIIA | 8,83 | 12 | 76,0 | 118,3 | 67,29 |
| 9 | M | 54 | IgG kappa | IIIA | 3,16 | 12 | 57,3 | 26,0 | 0,24 |
| 10 | M | 55 | IgG kappa | IIIB | 22,20 | 8 | 42,2 | 36,4 | 8,33 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 11 | M | 74 | lambda | IIIA | 7,00 | 3 | 44,3 | 23,0 | 0,06 |
| 12 | M | 45 | IgG kappa | IIIB | 9,10 | 8 | 50,7 | 46,7 | 0,41 |
| 13 | M | 58 | IgA kappa | IIA | 1,80 | 12 | 112,0 | 77,6 | 1,084 |
| 14 | F | 70 | IgG kappa | IIA | 4,90 | 8 | 44,0 | 14,5 | 0,79 |
| 15 | F | 45 | IgG kappa | IIIA | 2,60 | 8 | 42,0 | 29,8 | 0,55 |
| 16 | F | 65 | IgG lambda | IIIA | 7,30 | 12 | 68,0 | 56,7 | 0,38 |
| 17 | M | 76 | IgG lambda | IA | 2,12 | .8 | .52,0 | 52,5 | 0,66 |
| 18 | M | 55 | IgG kappa | IIB | 10,10 | 12 | 41,8 | 13,4 | 0,86 |
| 19 | M | 60 | IgG lambda | IIIA | 4,06 | 8 | 53,0 | 20,0 | 13,88 |
| 20 | F | 51 | kappa | IIIB | 75,70 | 12 | 80,7 | 73,6 | 66,67 |
| 21 | M | 44 | IgG kappa | IIIB | 17,40 | 12 | 83,8 | 65,4 | 4,83 |
| 22 | M | 81 | IgG kappa | IIIA | 5,30 | 12 | 126,5 | 23,2 | 1,67 |
| 23 | F | 75 | IgG lambda | IA | 2,60 | 8 | 36,6 | 52,4 | 3,70 |
| 24 | F | 57 | kappa | IIIB | 30,09 | 6 | 52,8 | 46,0 | 0,18 |
| 25 | F | 52 | IgD lambda | IIIA | 5,30 | 12 | 47,8 | 51,3 | 0,16 |
| 26 | F | 73 | Kappa | IIIA | 6,00 | 12 | 84,0 | 63,5 | 4,25 |
| 27 | M | 57 | IgA kappa | IIB | 9,10 | 6 | 24,6 | 24,3 | 0,54 |
| 28 | F | 67 | IgA lambda | IIA | 2,20 | 12 | 50,6 | 18,4 | 61,02 |
| 29 | F | 73 | IgA kappa | IIIA | 6,00 | 12 | 64,6 | 45,5 | 1,40 |
| 30 | F | 62 | IgD lambda | IIIA | 7,70 | 8 | 43,4 | 32,4 | 2,54 |
| 31 | F | 60 | IgG kappa | IIIB | 7,05 | 6 | 20,0 | 20,0 | 0,87 |
| 32 | F | 68 | kappa | IIA | 11,23 | 8 | 95,4 | 89,3 | 2,36 |
| 33 | F | 59 | IgG kappa | IIA | 3,40 | 12 | 52,8 | 57,0 | 1,44 |
| 34 | M | 64 | IgG kappa | IIA | 3,00 | 12 | 110,0 | 77,7 | 1,08 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 35 | M | 68 | IgA kappa | IIIA | 12,50 | 12 | 96,0 | 84,0 | 0,59 |
| 36 | F | 54 | IgG lambda | IIIA | 7,00 | 8 | 44,4 | 42,0 | 0,96 |
| 37 | F | 62 | IgA lambda | IIIB | 17,40 | 12 | 122,4 | 133,0 | 4,04 |
| 38 | M | 65 | lambda | IIA | 3,10 | 12 | 62,4 | 52,3 | 1,01 |
| 39 | M | 58 | IgG lambda | IIIB | 18,60 | 4 | 29,0 | 26,0 | 3,16 |

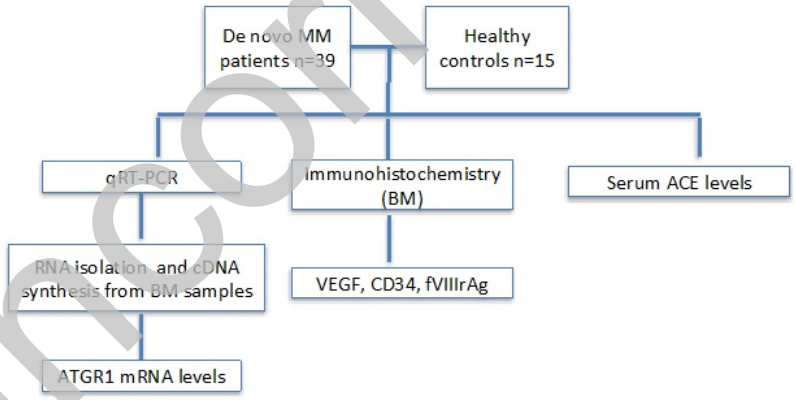
PP: paraprotein, B2M: beta 2 microglobulin (ng/ml), VEGF: vascular endothelial growth factor (score), CD34 & fVIIIrAg; # stained vessels/mm$^2$, AGTR1 mRNA: Angiotensin II type 1a receptor mRNA relative expression level (%).

Table 6: Angiogenesis factors and AGTR1 mRNA expression levels of the patients and controls

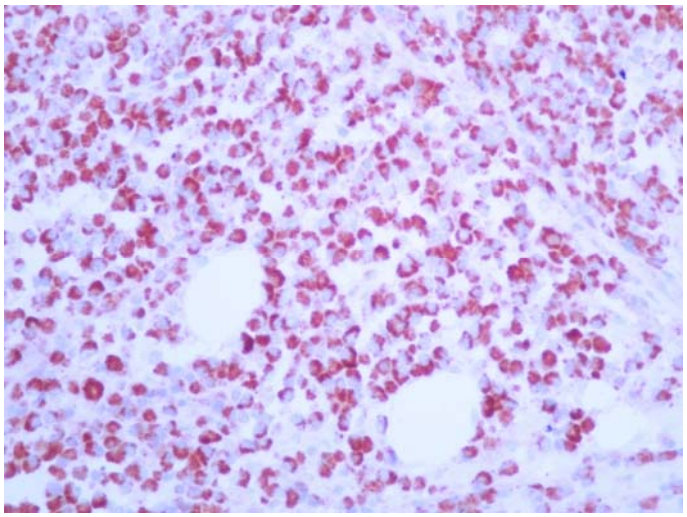
| | Patients | Controls | P value |
|---|---|---|---|
| VEGF index | 10±3 | 2±1 | <0.001 |
| CD34 (# of vessels) | 62.37±31.81 | 38.55±17.26 | 0.001 |
| factorVIIIrAg (u/ml) | 52.28±35.96 | 28.09±14.49 | 0.001 |
| AGTR1 mRNA | 6.82±17.22 | 1.17±1.09 | 0.04 |

Table 7: Angiogenesis factors and AGTR1 mRNA expression levels according to bone marrow infiltration pattern of the patients

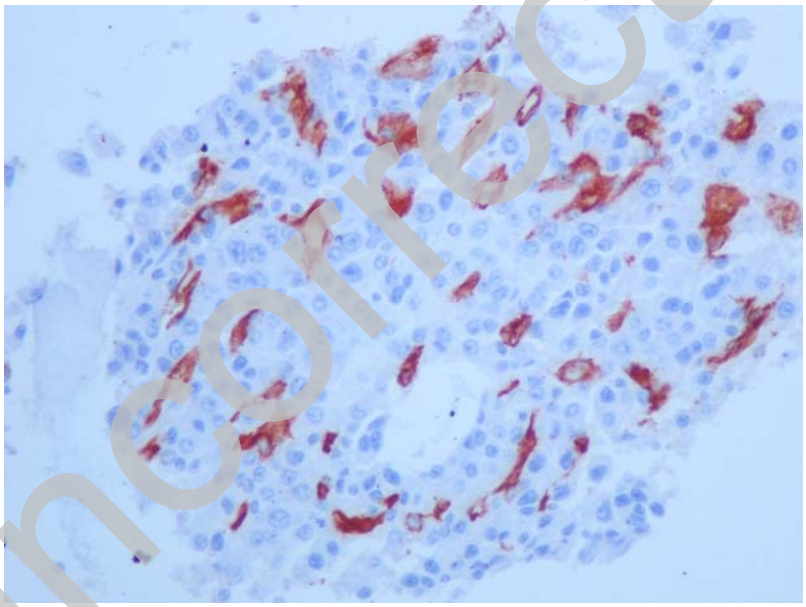
| | Mild-patchy infiltration | Severe-diffuse infiltration | P value |
|---|---|---|---|
| VEGF index | 8±3 | 10±2 | 0.017 |
| CD34 (# of vessels) | 47.24±24.92 | 69.36±32.61 | 0.045 |
| factorVIIIrAg (u/ml) | 37.16±21.44 | 59.25±39.39 | 0.032 |
| AGTR1 mRNA | 0.81±0.32 | 9.82±20.56 | 0.035 |



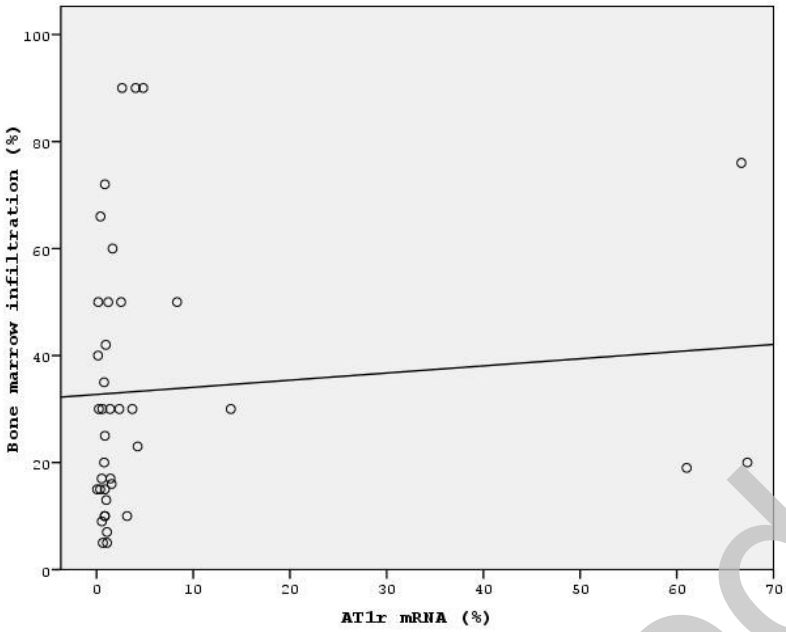

Flowchart of thr study



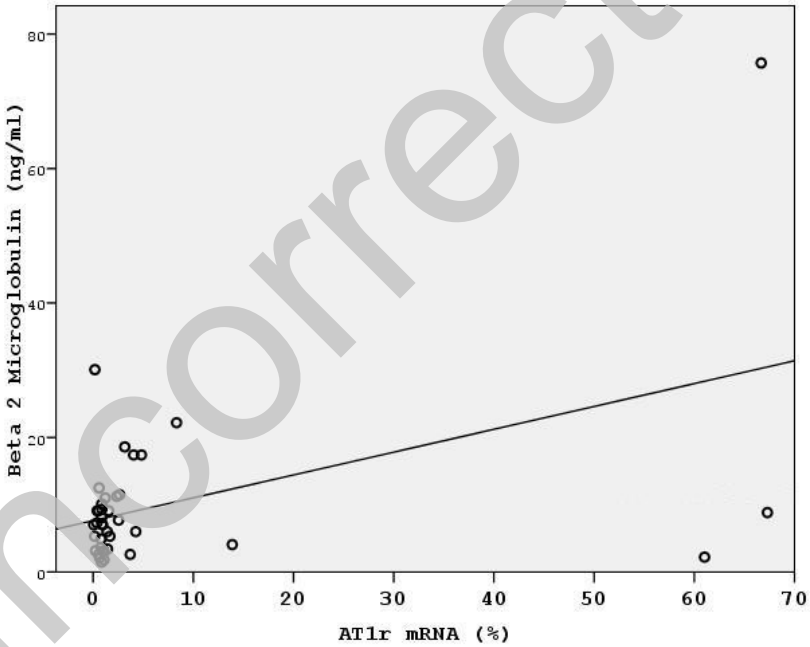
Bone marrow anti VEGF antibody, AEC chromogen, x400



Bone marrow anti CD34 antibody, AEC chromogen, x400




Correlation of the bone marrow infiltration ratio and AGTR1 mRNA expression of the Patients




Correlation of the bone marrow AGTR1 mRNA expression and serum beta-2 microglobulin level of the patients